ASPERGILLUS -SPECIFIC HOST RESPONSES AND T HELPER DEFICIENCIES IN CLINICAL SETTINGS

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Aspergillus-specific host responses and T helper deficiencies in clinical settings

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General introduction and outline of the thesis

GENERAL INTRODUCTION

Aspergillus is a mould fungus found ubiquitously in the environment, but most abundantly in soil and water. It continuously forms spores, which become airborne once they came into contact with the surrounding air and are dispersed easily due to their high hydrophobicity [1-3]. We inhale several hundred conidia per day that continuously interact with our immune system [2, 4]. We evolved a sophisticated host defence system that both controls and tolerates the fungal burden [5]. A dysregulated host response towards *Aspergillus*, either being hyper- or hypo-reactive, is the cause for developing *Aspergillus*-related disease [6].

THE *ASPERGILLUS* SPECIES

Many species belong to the *Aspergillus* family, but the species *A. fumigatus* is the most prevalent disease-causing agent [3]. Many factors contribute to this fact: *A. fumigatus* grows and survives under a wide range of environmental conditions [7], it adapts very efficiently to the nutrients in immunocompromised hosts and is able to colonize the lung of allergic patients; due to their size the conidia (2-3 µm diameter) can reach the alveoli easily [3, 8-10]. Interestingly, in allergic and invasive pulmonary aspergillosis the prevalence of different *Aspergillus* spp. is quite similar. While *A. fumigatus* is the most commonly isolated strain followed by *A. flavus*, species like *A. niger, A. terreus* or *A. nidulans* are much less prevalent [11-13]. Studying the *Aspergillus*-host-interaction requires appreciation of both the pathogen and the host cell(s) it encounters. The cell wall of the *Aspergillus* conidium consists of an outer hydrophobic rodlet layer and an inner polysaccharide core, which can biochemically be separated into an alkali-insoluble fraction containing β-(1,3)-glucan covalently bound to chitin and galactomannan (GM) and an alkali-soluble fraction composed of a linear chain of α-(1,3)glucan linked GM

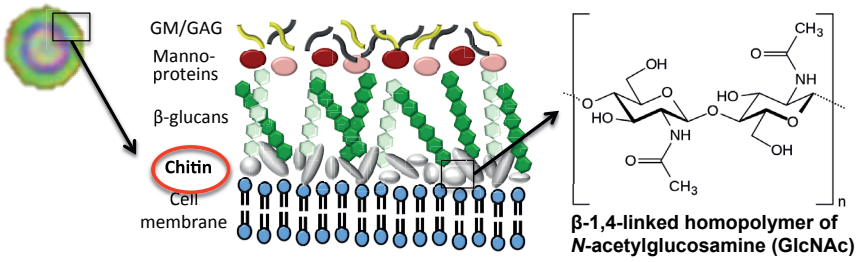

Figure 1: *The polysaccharide cell wall of the Aspergillus conidium with special attention to chitin*

or galactosaminogalactan (GAG) [14] (Figure 1). The rodlet layer shields the resting *Aspergillus* conidium from recognition by the immune cells [15]. Once the different types of polysaccharides are exposed, their interaction with the immune system differs [14]: while a pro-inflammatory response has been associated with β-(1,3)-glucan recognition, an anti-inflammatory function has been described for GAG [16, 17], and both anti- and pro-inflammatory responses have been described for chitin [18-21].

FROM INNATE TO ADAPTIVE IMMUNE RESPONSES IN ASPERGILLOSIS

Once an *Aspergillus* conidium is inhaled, a whole network of host cells consisting of lung epithelial cells, macrophages, NK cells and neutrophils will respond fast, but nonspecifically to the invading fungus. Conserved structures present on invading microorganisms are recognized by pattern recognition receptors (PRRs), and activation of subsequent signalling pathways leads to secretion of innate cytokines that initiate the differentiation of naïve T cells towards T helper cells. PRRs allow innate immune cells to initiate different responses dependent on the microorganism it encounters, a concept that was first postulated in 1989 by Charles Janeway [22]. In 1991 the *Toll* gene was found to regulate anti-fungal immunity in *Drosophila* [23], and in 1996 Toll-like-receptors (TLRs) were described in humans as the first class of PRRs, discoveries for which the Nobel prize was awarded in 2011 [24, 25]. Over decades several other classes of PRRs such as NOD-like receptor (NLRs), C-type lectins receptors (CLRs) and RIG-I-like receptors (RLRs) have been discovered. Once those receptors have regognized the corresponding pathogen associated molecular pattern (PAMP) downstream signalling cascades consisting of various kinases or adapter molecules get actived. For example, the dectin-1 receptor signals after binding of β-(1,3)-glucan via Syk kinase and the adapter molecule CARD9. Activation of the transcription factor NF-κB results in transcription of genes encoding for pro-inflammatory cytokines, such as pro-IL-1β. Once a second signal is provided the inflammasome containing the caspase-1 gets activated and pro-IL-1β is cleaved into the active IL-1β. This pro-inflammatory cytokine machinery underlies a tight regulation to prevent from overshooting cytokine stimulation. Crosstalk and co-signalling between different receptors and pathways can explain the variety of different types of immune responses. Via this mechanism fungal PAMP binding the corresponding PRR can direct the adaptive immune response [26]. Although less fast, the initiation of a specific and durable adaptive immune response is crucial for successful clearance of the pathogen. After recognition of *Aspergillus* by cells of the innate immune system such as alveolar macrophages, cytokines produced by innate cells will lead to the differentiation of naïve CD4+ cells into different T helper (Th) subsets (Figure 2).

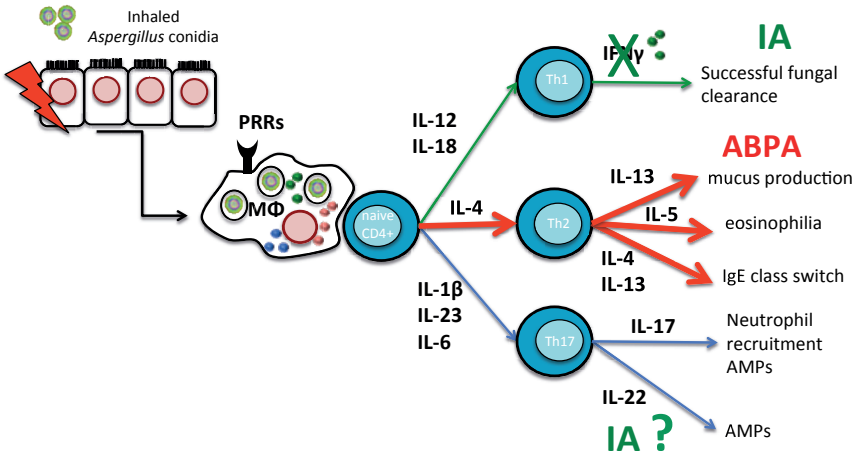

Figure 2: *Schematic overview: from innate to adaptive host defence in aspergillosis. Deficiency due to Th1 and possibly Th17 leads to invasive aspergillosis (IA), while overwhelming Th2 reaction can cause allergic bronchopulmonary aspergillosis (ABPA). AMPs (antimicrobial peptides).*

Plasticity of immune cells is an efficient mechanism of the immune system to adapt to different conditions quite rapidly. It has been shown that the type of cytokines present in the environment determines the direction of the differentiation of the cell, resulting in the Th subsets Th1, Th2, Th17, Th22 or Th9, some of them having plasticity [27].

IL-12 and IL-18 initiate the differentiation towards Th1; a strong Th1 response has been associated to be protective for invasive aspergillosis [28]. In contrast, IL-4 triggers Th2 differentiation characterized by the production of IL-4, IL-5 and IL-13, cytokines that have been shown to drive pathology in allergic bronchopulmonary aspergillosis by inducing mucus production, mast cell activation and eosinophilia [29]. A strong Th2 response is detrimental for successful fungal clearance [28]. Less clear is the role of the more recently described Th17 cells. In the presence of IL-1β and IL-23, Th cells can differentiate into Th17 cells that are characterized by the production of the cytokines IL-17 and IL-22. On the one hand, Th17 cells were shown to mediate antifungal control at the site of the mucosa; on the other hand they have been associated with harmful effects [30]. In addition, another distinct subpopulation of $CD4^+$ T helper cells, the Th22 cells characterized by their production of IL-22 and TNFα, play an important role in mucosal host defence [31]. Another more recently described T helper subset are Th9 cells: in the presence of IL-4 and TGFβ a naïve T cell differentiates into a Th9 cell, which promotes allergic inflammation [32, 33]. Interestingly, also Th17 cells have been described to be a source for IL-9. TGFβ and IL-1β can induce both IL-9 and IL-17 in T cells that play an important role in driving pathology in autoimmune diseases [34, 35]. Moreover, in this context, the

regulatory T cells are an important counterpart suppressing Th1, Th2 and Th17-mediated inflammation in the *Aspergillus*-specific host response.

CLINICAL SYNDROMES ASSOCIATED WITH *ASPERGILLUS* AND OTHER OPPORTUNISTIC PATHOGENS

As already mentioned, a healthy immune system can easily handle the tremendous amount of inhaled *Aspergillus* conidia. Patients with underlying diseases severely affecting the immune response are prone to develop fungal diseases or other opportunistic infections. The clinical spectrum of *Aspergillus*-associated diseases ranges from allergic bronchopulmonary aspergillosis (ABPA), an allergic reaction in a chronically colonized lung, to localized infections such as chronic pulmonary aspergillosis or to invasive aspergillosis (IA), [36] (Figure 3).

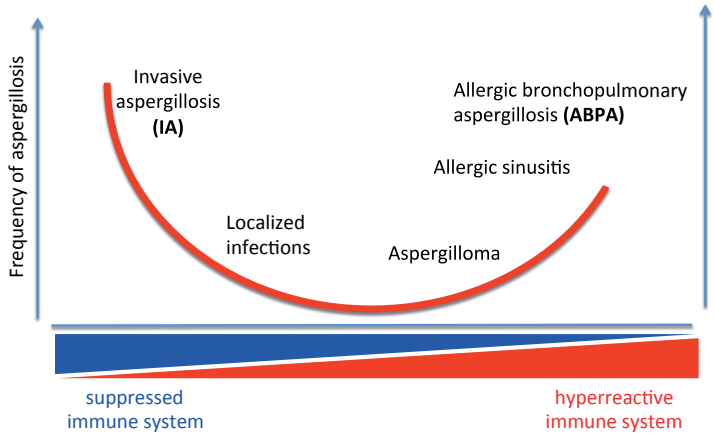

Figure 3: *Clinical spectrum of Aspergillus-associated diseases (adapted from Denning et al. Clinical and Translational Allergy 2014)*

ABPA

Fungal pathogens have been linked to allergy causing agents for the first time by Storm Van Leeuwen in 1925 [1] and cases with ABPA have been reviewed for the first time 1952 by Hinson *et al.* [37]. A hypersensitive reaction to *Aspergillus* is the core immunopathological mechanism underlying ABPA. While asthma with fungal sensitization affects roughly 33% of severe asthma patients, ABPA is less common. Around 10% of cystic fibrosis (CF) patients and 2% of asthma patients suffer from APBA [38]. The typical

diagnostic criteria of ABPA comprise asthma, immediate skin reactivity to *Aspergillus* antigens, serum IgE > 1000 ng/ml and facultative central bronchiectasis [39]. Mainstays of the therapy are a general immunosuppressive treatment with oral administration of corticosteroids next to antifungal therapy with itraconazole, which has been shown to be beneficial for ABPA patients [40].

LOCALIZED *ASPERGILLUS* INFECTIONS

Especially pre-formed cavities such as bronchiectases in the lung or the sinus of the skull base are preferential localisations for localized *Aspergillus* infections, such as aspergilloma in the lung or skull base osteomyelitis [36, 41]. As long as *Aspergillus* does not invade surrounding blood vessels, theses infections can present without any symptoms. Otherwise haemoptysis or headache and nerve palsy, respectively, are common symptoms. These patients are not generally immunocompromised, but rather have specific immune defects with the consequence that aspergillomas can exist not rarely for over years before being noticed [36].

INVASIVE ASPERGILLOSIS

Neutropenia is the most common risk factor for the development of IA especially affecting patients, who underwent an organ or bone-marrow transplantation [42]. Diagnosis remains challenging, since fungal cell wall components measured in the blood, such as galactomannan and β-glucan, are difficult to detect during the early stage of disease and immunological parameters are often unreliable due to the severe immunosuppression in patients that develop IA [43]. Despite early and adequate treatment with Amphotericin B or azoles the mortality of IA remains very high, ranging from 30% - 60% depending on the grade of immunosuppression and the grade of dissemination of the IA [42].

CANDIDIASIS

Aspergillus is not the only fungal pathogen that has been associated with allergic syndromes and infections in immunodeficient patients: a whole range of different fungal species other than *Aspergillus* can lead to allergic bronchopulmonary mycosis or invasive infections [44]. Also clinical syndromes presenting with *Candida* infections that are associated with allergic phenotype are known. *Candida* is a commensal of the body and an opportunistic pathogen. It can cause infections in immunocompromised patients such as patients undergoing organ or bone marrow transplantation or suffering from

haematological malignancies [45]. Furthermore, another group of patients has recently been described to be on high risk to develop mucosal *Candida* infection: patients with an IL-17 deficiency [46]. Patients with a *loss-of-function* mutation in the *STAT3* gene, leading to lower activity of RORγT, the transcription factor mediating the differentiation of a naïve CD4 cells towards a Th17 cell, suffer from hyper IgE syndrome (HIES). Clinically, they present with a higher susceptibility for infections, such as recurrent staphylococcal skin or pulmonary infections as well as chronic mucocutaneous candidiasis (CMC), but also with an allergic phenotype; they suffer from eczema, eosinophilia and high IgE serum levels [47]. Another cause of CMC, a *gain-of-function* mutation in the *STAT1* gene leading to a STAT1 hyperphosphorylation and complete lack of IL-17 producing cells, has recently been identified [48, 49]. In contrast to the HIES patients, CMC patients do not present with an allergic phenotype.

NON-TUBERCULOUS MYCOBACTERIA

The lung of patients with cystic fibrosis (CF) is often colonized with *Aspergillus* as well as with opportunistic bacteria, such as non-tuberculous mycobacteria (NTM) [50]. Similar to *Aspergillus,* NTMs are ubiquitously in soil and water and infections are commonly associated with underlying immunodeficiencies. Especially defects affecting the IFNγ-signalling, such as polymorphisms in the IFNγ, IL-12 or STAT1-pathway or even autoantibodies against IFNγ cause a high susceptibility to NTM infections [51]. In addition to CF patients, patients with the Lady Windermere syndrome are at high risk to develop NTM infections. These patients present with a unique marfanoid body phenotype and demonstrate a skewed immune response with lower IFNγ and TGFβ levels [52, 53]. There are interesting clinical and immunological parallels between these clinical syndromes associated with opportunistic fungal and mycobacterial infections.

In conclusion, clinical presentations caused by fungal and other environmental opportunistic pathogens are mainly determined by the immunological status of the host. Deviations from the normal status into either a hyper-reactive or an immunocompromised status are associated with allergic or invasive aspergillosis, respectively. Little is known about the exact underlying immunopathology. The aim of this thesis is to use a multidisciplinary approach and to combine genetic, immunological and clinical knowledge to increase our knowledge of the dysregulated pathways and immunophenotypes associated with these diseases.

OUTLINE AND AIMS OF THE THESIS

Fungus associated molecular patterns and human pattern recognition receptors have a continuous interplay that is essential for preventing infections. In this thesis I aim to identify the recognition pathways and T helper responses involved in the host response in allergic and invasive aspergillosis, as well as opportunistic infections often associated with an altered immune status. By studying genetic polymorphisms as well as immunological mechanisms underlying the various opportunistic infections we can gain knowledge to develop more target-specific immune-modulatory therapies to supplement conventional treatment regimens that are often limited in their effectiveness and accompanied with severe side-effects.

ASPERGILLUS RECOGNITION AND INITIATION OF T HELPER RESPONSES

Once an *Aspergillus* conidium is inhaled it first encounters the innate immune cells of the respiratory tract. Many studies have been performed to identify pattern recognition receptors and different cell populations orchestrating the antifungal immune response to efficiently clear the invading pathogen. How these receptors and cells form a complex network in the *Aspergillus*-specific immune response is reviewed in **Chapter 2**.

To successfully clear the fungal invaders the first step is the successful recognition of fungal PAMPs by human PRRs. Polysaccharides are crucial components of the fungal cell wall. While for many of them, such as β-glucans and mannose, human PRRs had been identified, there is controversy about the receptor(s) recognizing the polysaccharide chitin. On the one hand chitin has been described not to be immunogenic [21], on the other hand a profound anti-inflammatory capacity mediated via mannose receptor and TLR9 [18] has been attributed to chitin. Since few studies have been performed in human cells to date, we studied the immunological properties and PRRs of *Aspergillus* chitin in the human context, see **Chapter 3**.

Following the innate immune response against *Aspergillus,* specific adaptive cytokine combinations are induced which stimulate different Th responses. Thereby, different PRRs trigger different Th responses, which are again associated with a different clinical presentation of aspergillosis. A strong Th1 response has been associated with a successful fungal clearance and Th17 responses represented by the production of IL-17 and IL-22 were associated with either detrimental or protective effects. In contrast, elevated Th2 responses are associated with allergic symptoms and *Aspergillus* is a potent inducer of allergic reactions causing ABPA. Which receptors, lymphocyte subsets and regulating cytokines are involved in the Th1 and Th17 responses, is displayed in **Chapter 4**.

To characterize the whole range of Th responses in aspergillosis we studied pattern recognition pathways leading to *Aspergillus*-specific Th2 responses. In **Chapter 5**, we investigated the PRRs leading to a Th2 cytokine bias of the *Aspergillus*-specific host response, and we characterized the Th cytokine profile of ABPA patients compared to healthy controls and asthma patients sensitized with *Aspergillus*.

Several fungal infections have been associated with mutations in genes encoding for anti-fungal immune responses that result in dysregulation of certain cytokines. The search for unknown genetic variations can be crucial to better understand fungal diseases. Only a few studies have been performed to date, which identify the genetic background in ABPA patients [54]. Therefore, we have studied the genetic background of patients with ABPA in an Indian cohort and compared this genetic background with patients with asthma, as well as with healthy controls from the same geographic region (**Chapter 6**).

After successful recognition, *Aspergillus* conidia are phagocytosed and intracellularly killed in phagolysosomes mediated by the low pH and a high concentration of reactive oxygen species (ROS) produced by the NADPH-oxidase. Patients with chronic granulomatous disease (CGD), who lack the NADPH-oxidase, are at great risk for *Aspergillus* infections [55]. Although *A. fumigatus* is the most commonly isolated *Aspergillus* species in CGD patients, *A. nidulans* is much more prevalent in these patients compared to other immunocompromised patients, e.g. after stem cell transplantation. In **Chapter 7**, we aim to identify differences in phagocytosis and killing as well as in the induction of cytokines and ROS molecules between *A. fumigatus* and *A. nidulans*, which might help to explain the higher prevalence of *A. nidulans* in CGD patients.

T HELPER DEFICIENCIES IN CLINICAL SETTINGS

Aspergillus typically affects the lung and only rarely the bone and even less commonly the skull base. While IA or ABPA patients usually have an immunocompromised or hyperreactive immune response, respectively, the immune defect in patients with *Aspergillus* skull base osteomyelitis (SBO) was unknown. In **Chapter 8**, we investigated whether these patients demonstrate a certain immunological profile that would explain their higher susceptibility to *Aspergillus* skull base infections.

In addition to ABPA there are more fungus-related immunological disorders associated with an allergic phenotype, such as HIES. In contrast to ABPA patients, who present with the classical asthmatic symptoms caused by *Aspergillus* allergens, in HIES patients with a *STAT3 LOF* mutation, the skin is primarily affected. They present on the one hand with allergic skin symptoms such as atopic features, namely eczema, eosinophilia and

high IgE levels, but also with skin infections, such as chronic mucocutanous candidiasis and staphylococcal skin abscesses [47]. These patients demonstrate a deficient Th17 response, which is also a characteristic feature of CMC patients with a *STAT1 GOF* mutation. HIES and CMC have very similar clinical phenotypes and underlying immunological defects. In **Chapter 9**, we compare the allergic immune responses against *Aspergillus, Candida* and *S. aureus* of HIES and CMC patients to investigate how in the setting of an IL-17 deficiency the allergic Th2 and Th9 responses are modulated and whether there would be differences that might explain an allergic phenotype in HIES which is not present in CMC.

Opportunistic pathogens cause pulmonary infections only in patients with immunological deficiencies. Similar to *Aspergillus*, non-tuberculous mycobacteria (NTM) can cause chronic colonisation and pulmonary infection in immunodeficient patients and they are often present together, most obviously in patients with CF. In contrast to a healthy lung, the lungs of CF patients has a very diverse microbiome consisting of environmental pathogens such as *Aspergillus* and NTMs. The majority of CF patients is chronically colonized with *Aspergillus*, which leads to the full clinical picture of ABPA in 10% of the cases [38]. In addition, the CF lung is chronically colonized with NTMs [56]. However, a strict distinction between infection and colonisation is difficult. Similar pathways in cytokine signalling have been associated with both fungal as well as NTM-associated diseases, especially IFNγ-signalling defects [57]. We therefore investigated immune responses of different patient groups with NTM infection. In **Chapter 10**, we compared the immunological profile of three CF patients infected with *M. abscessus* with a group of non-CF patients with pulmonary NTM infections to rule out whether the CF patients demonstrate deficiencies in *M. abscessus*-specific Th responses that might explain their higher susceptibility to *M. abscessus*.

NTM infections are not only seen in patients with CF, but have been reported more frequently from patients exhibiting a unique body phenotype with elongated fingers, scoliosis and pectus excavatum comparably to patients with Marfan syndrome. This phenotype is called 'Lady Windermere syndrome' [53]. In **Chapter 11**, we investigated the possible genetic cause for the syndromal physical phenotype and the failure in the clearance of NTM infections. We also studied whether these patients demonstrate a distinct cytokine production compared to healthy controls. We performed whole-exome-sequencing in 11 patients and their family members and performed functional validation assays.

Finally, a summary of the new findings and a conclusion of this thesis are presented in **Chapter 12**.

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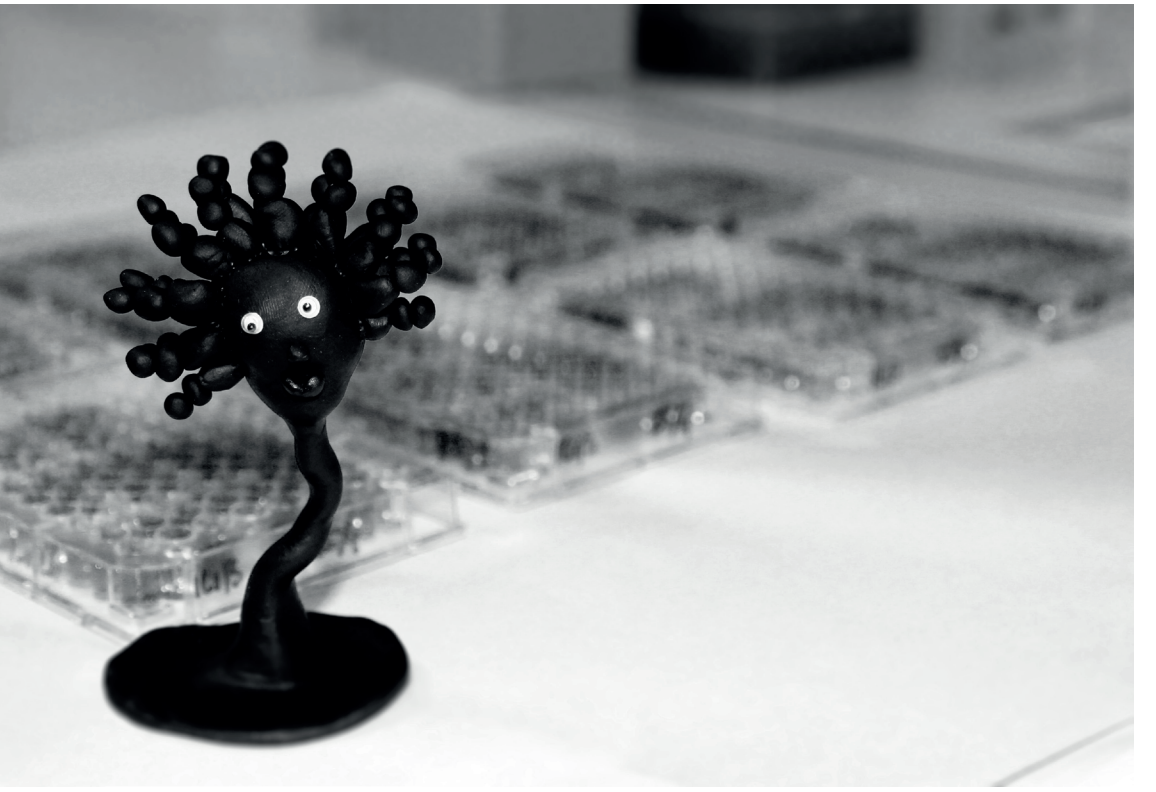

CHAPTER 2

Anti-fungal innate immunity: recognition and cellular networks

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ABSTRACT

A large variety of fungi are present in the environment, among which a proportion colonizes the human body, usually without causing any harm. However, depending on the host immune status, commensals can become opportunistic pathogens that induce diseases ranging from superficial non-harmful infection to life-threatening systemic disease. The interplay between the host and the fungal commensal flora is being orchestrated by an efficient recognition of the microorganisms, which in turn ensures a proper balance between tolerance of the normal fungal flora and induction of immune defence mechanisms when invasion occurs. Pattern recognition receptors (PRRs) play a significant role in maintaining this balance due to their capacity to sense fungi and inducing host responses such as the induction of proinflammatory cytokines involved in the activation of innate and adaptive immune responses. In the present review we will discuss the most recent findings regarding the recognition of *C. albicans* and *A. fumigatus* and the different types of immune cells that play a role in antifungal host defence.

INTRODUCTION

During the last few decades the prevalence of fungal infection has considerably increased, especially due to the immunosuppressive treatments in cancer therapy, transplantation, and the HIV epidemics (1). Moreover, pathogenic fungi are able to rapidly adapt and become resistant to antifungal agents (2). The human commensal *Candida albicans* and the environmental fungus *Aspergillus fumigatus* are facultative pathogens, which in immunocompetent individuals do not cause infection. However, immunocompromised patients are prone to systemic fungal infections in which mortality rates can rises up to 60% (3). While the clinical presentation of candidiasis and aspergillosis differs, the mechanisms needed to control infection including the pattern recognition and inflammatory networks share many characteristics.

PATTERN RECOGNITION RECEPTORS IN FUNGAL INFECTION

Both *Candida* and *Aspergillus* undergo a continuous morphology switch between yeasts and hyphae, resulting in differential exposure of fungal pattern associated molecular patterns (PAMPs). *C. albicans* cell wall contains carbohydrate polymers and glycoproteins, comprising mainly chitin, β-1,3- and β-1,6-glucan and *O*- and *N*-linked mannan. The *Aspergillus* cell wall is predominantly composed of chitin, α-glucans, β-(1,3)-(1,4)-glucans, and galacatomannans. One important feature of the *Aspergillus* conidial cell wall is the outer layer of hydrophobic rodlets. During swelling of the conidial cell wall and subsequent germination, the hydrophobic layer changes into a hydrophilic structure exposing polysaccharides. During the course of fungal infection and invasion, the first crucial step in mounting effective immune responses is the recognition of the invaders by the host. Various PRRs on differential cell populations will recognize fungal invaders and trigger bona fide signalling pathways and cellular responses (Figure 1).

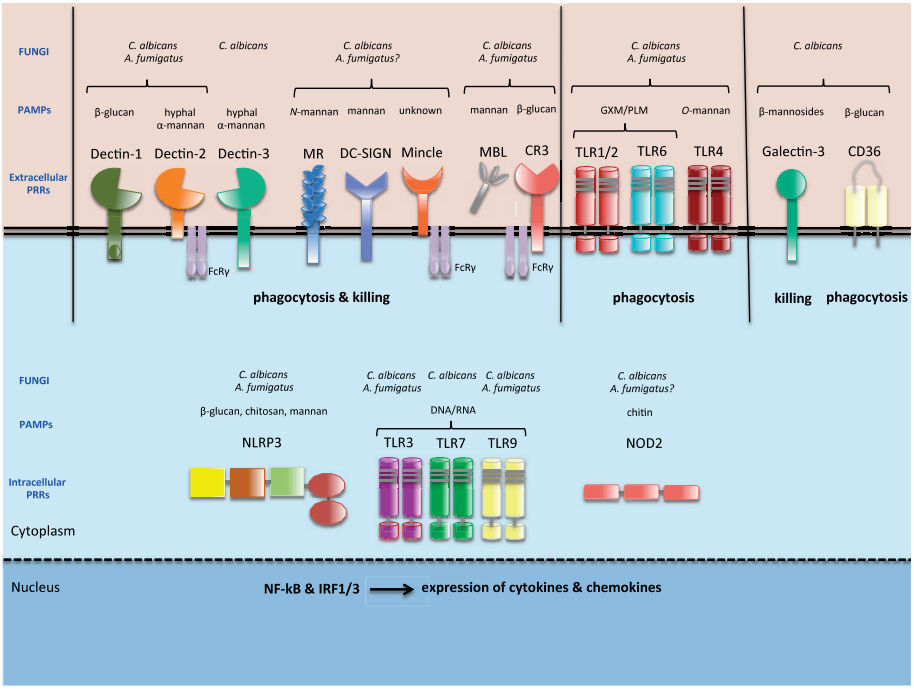

Figure 1: Recognition of *C. albicans* and *A. fumigatus* by innate immune cells

The pattern recognition receptors Dectin-1, Dectin-2, Dectin-3, FC gamma receptor (FcRγ), Mannose Receptor (MR), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN), Mincle, Mannan-Binding Lectin (MBL), Complement Receptor 3 (CR3), Galectin-3, Cluster of Differentiation 36 (CD36), Toll-Like Receptors (TLRs), NACHT, LRR, and PYD domain–containing protein 3 (NLRP3), and Nucleotide-binding Oligomerization Domain–containing protein 2 (NOD2) recognize the fungal cell wall components β-glucan, α-mannan, β-mannan, *N/O*-mannan, phospholipo-mannan (PLM), glucuronoxylo-mannan (GXM), chitosan, chitin, or fungal DNA and RNA. These signals activate canonical or non-canonical nuclear pathways, with the subsequent induction of an immune response, such as secretion of cytokines, chemokines, initiation of phagocytosis or inflammasome activation.

C-type lectin receptors (CLRs)

Mannans present in the outer layer of *Candida* and *Aspergillus* cell wall come in direct contact with the host and can be recognized by the C-type lectin receptors (CLRs). Mannose receptor (MR), primarily present on macrophages, recognizes *Candida N*-mannan (4), and further induces IL-17 (5). MR has also been shown to recognize *Aspergillus* conidia and induces proinflammatory cytokines (5). Dectin-2, which is mainly expressed on DCs, macrophages and neutrophils recognizes *Candida* α-mannan and also triggers IL-17 (6). In addition to its role in modulating Th responses, Dectin-2 has also been associated with ROS production, as well as phagocytosis and killing of *C. glabrata* (7).

Dectin-2 forms heterodimers with Dectin-3, leading to proinflammatory responses upon *C. albicans* infection (8). Dectin-2 is also important for the *Aspergillus*-specific immune response, being upregulated upon recognition of the fungus. Its expression is restricted to the population of monocyte-derived macrophages (9).

Galectin-3 on macrophages recognizes β-mannans (10), and induces a protective anti-fungal response in murine macrophages through the secretion of TNFα. Moreover, mice deficient in galectin-3 are more susceptible to disseminated candidiasis (11). Mincle is a CLR expressed on monocytes and neutrophils, yet with an unknown ligand. In murine models Mincle was responsible for inducing protective responses against *C. albicans*, mainly by the initiation of TNFα production; mice deficient in this receptor were drastically susceptible to systemic candidiasis (12). However, the role of Mincle in *Aspergillus* recognition is still unknown. DC-SIGN is present on dendritic cells as well as macrophages, and recognizes *Candid*a *N*-linked mannan (13), and can bind and internalize *Aspergillus* conidia (14). It induces a cascade of cytokines that activate T helper cells in order to differentiate and initiate adaptive immune responses. Mannose-binding lectin (MBL) is a soluble CLR that also binds mannan modulating the recruitment of phagocytes and proinflammatory responses against *Candida* (15).

β-1,3- and β-1,6-glucan is shielded by the mannoproteins in *C. albicans* yeast, but it is exposed on budding yeast, and *Candida* hyphae. Dectin-1, a well-described CLR present on macrophages and monocytes, binds to a long β-1,3-glucan chain (16) inducing cytokines as well as internalization of the fungus upon formation of a "phagocytic synapse" (17). Resting *Aspergillus* conidia do not present abundant β-glucans on their surface either, which might account for the redundant role of Dectin-1 in induction of different Th subsets in experimental *in vitro*-models (18, 19). Inhibition of Dectin-1 on alveolar macrophages did not affect phagocytosis of resting *A. fumigatus* conidia (20). In contrast, alveolar macrophages stimulated with germinated conidia produce innate cytokines via the Dectin-1 pathway (21). Dectin-1 was described to be involved in both clinical spectra of aspergillosis: in an invasive aspergillosis mouse model, IL-17 production was mediated by Dectin-1 and was dependent on IL-23 (22); in an allergic mouse model Dectin-1 was demonstrated to drive pulmonary pathology via IL-22 production (23). Corticosteroids blocked *Aspergillus*-induced autophagy via the Dectin-1/Syk pathway (24), and corticosteroid resistant asthma was associated with detrimental IL-17 and IL-22 production (25), suggesting a strong role for autophagy and Dectin-1 to drive pathology in severe forms of asthma. Dectin-1 polymorphisms were not associated with disseminated candidiasis in humans, but were associated with *Candida* colonization, while susceptibility to invasive aspergillosis was dependent on dectin-1 polymorphisms (26, 27). In conclusion, CLRs

play an important role in mediating immunity against *Candida* and *Aspergillus* through inducing Th derived cytokines and mediating uptake of the fungus.

Toll like receptors (TLRs)

TLRs have been extensively studied in the context of fungal recognition. TLRs play a significant role in a murine model of disseminated candidiasis and together with IL-1 receptor activate several potent antifungal-signalling mechanisms (28). TLR2$^{-/-}$ mice were reported to have an increased susceptibility to disseminated candidiasis that could be attributed to a decreased secretion of TNFα and MIP-2 and impaired recruitment of neutrophils. In contrast, others have shown that TLR2$^{-/-}$ mice are more resistant to systemic candidiasis exhibiting an increased chemotaxis and IFNγ secretion, while IL-10 was significantly impaired. Interestingly, the recruitment of monocytes was enhanced in TLR2$^{-/-}$ mice, suggesting an increase in the candidacidal activity in these mice (29). TLR4$^{-/-}$ mice were more susceptible to systemic candidiasis due to an impaired secretion of KC and decreased neutrophil recruitment resulting in a higher fungal burden in the kidneys (30). However, TLR1 polymorphisms, but not TLR2 and TLR4 polymorphisms have been associated with human susceptibility to *C. albicans* infection (27). TLR4$^{-/-}$ mice were more susceptible to *Aspergillus* infections and TLR4 polymorphisms were associated with invasive aspergillosis (28, 31). Immunosuppression of TLR2$^{-/-}$ mice resulted in higher susceptibility to *Aspergillus* infections (32), however polymorphisms in TLR2 were not associated with invasive aspergillosis (26). The intracellular receptor TLR3 recognizes fungal nucleic acids. TLR3 had a protective role against *Candida* as a mutation in the L412F variant of TLR3 led to lower activation of NF kB and decreased levels of IFNγ, resulting in increased susceptibility to cutaneous candidiasis (33). TLR3$^{-/-}$ and TRIF$^{-/-}$ mice were found to be susceptible to invasive pulmonary aspergillosis, and TLR3 polymorphisms have been associated with invasive aspergillosis (26, 34), suggesting an important role of TLR3 in mediating protection against *Aspergillus* infection as well. Conclusions on the role of TLR7 and TLR9 in fungal infection have not been consistent, and no polymorphisms in TLR7 and TLR9 have been associated to date with invasive aspergillosis or candidiasis (26, 27).

NOD-Like receptors (NLRs)

NOD1 and NOD2 are intracellular NLRs that recognize bacterial peptidoglycans. Chitin-mediated responses, such as the induction of IL-10, were found to be dependent on NOD2 as well as TLR9 and MR (35). However, there is presently only *in-vitro* data to support a role for NOD2 in fungal infection, no *in-vivo* studies in NOD2 mice have been performed and no studies have been performed on the role of NOD1 in fungal infection.

The best-characterized NLR is NLRP3, which is a component of the inflammasome. Mice deficient in NLRP3 were more susceptible to *Candida* infections (36-38). One study showed involvement of NLRP3 in anti-*Aspergillus* host defence *in-vitro*, however disseminated aspergillosis has not been studied in NLRP3 deficient mice (39).

INFLAMMATORY NETWORKS

Recognition of fungal associated molecular patterns by PRRs will lead to the initiation of an efficient host defence that is mandatory to clear the invading fungus. Several populations of cells are involved that orchestrate this clearance (Figure 2).

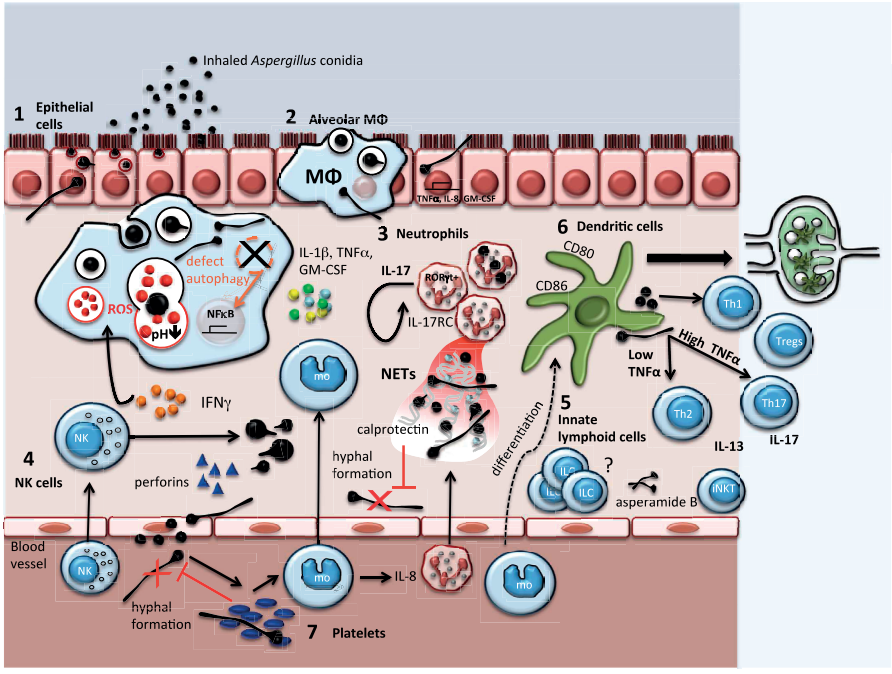

Figure 2: Inflammatory networks of the innate immune response against *Aspergillus*

1) Inhaled *Aspergillus* conidia reaching the alveoli, are endocytosed by the lung *epithelial cells*, in which they survive or escape from via hyphal formation. Epithelial cells react upon fungal growth with expression of pro-inflammatory cytokines. 2) Once internalized in *alveolar macrophages and monocytes, Aspergillus* conidia are phagocytosed, which is prevented by hyphal formation. Acidification in the phagolysosomes is essential for the killing of *A. fumigatus* conidia. Reactive oxygen species (ROS) are produced to effectively kill and eradicate *Aspergillus*. A defect ROS system of macrophages and monocytes like in CGD is associated with defect autophagy resulting in pro-inflammatory cytokine production. 3) Upon invasion, *neutrophils* are recruited

by pro-inflammatory cytokines produced by macrophages and monocytes or by IL-17 expressed in RORγt+ neutrophils and acting on the IL-17RC receptor via an autocrine feed-back loop. *Aspergillus* conidia are intracellular killed by the ROS system, while *Aspergillus* conidia and hyphae bind to neutrophils extracellular traps (NETs), which mainly consist of neutrophil DNA and antimicrobial proteins like calprotectin, inhibiting the fungal growth without killing. 4) *Natural killer (NK) cells* are activated by the germinated and hyphal forms of *Aspergillus* and mediate the killing via perforin release or directly via IFNγ. Next to that, IFNγ enhances conidial killing by macrophages. 5) The role of *innate lymphoid cells (ILCs)* in the lung during invasive aspergillosis is largely unknown. Invariant (i) NKTcells are directly activated by asperamide B derived from *Aspergillus* and cause together with IL-13, derived from Th2 cells, allergic airway responses. 6) Monocyte-derived *dendritic cells (DC)* recognize *Aspergillus* conidia and hyphae resulting in upregulation of CD80+ and CD86+ and the production of IL-8 and IL-12p70. DCs shape adaptive Th responses after migration to the lymphnode. *Aspergillus* conidia induce preferentially, Th1/Treg responses and *Aspergillus* hyphae Th17/Th2 responses. DCs, high positive for TNF, mediate differentiation into the Th17 direction with production of IL-17 and low positive for TNF into the Th2 direction with production of IL-13. 7) As last step of invasion *Aspergillus* can overcome the endothelium and reach the blood stream. All growth forms of *Aspergillus* activate *platelets*, which activate monocytes to produce IL-8. Activated platelets are capable to inhibit hyphal formation.

Epithelial cells

The epithelium constitutes a mechanical barrier against invading fungi. *C. albicans* is in permanent contact with epithelial cells and infection with tissue invasion is associated with morphological changes of yeast into hyphae. *Candida* hyphae can penetrate the epithelial cells through two distinct mechanisms: induced endocytosis and active penetration. Epithelial cells respond to *Candida* with activation of NFκB and cytokine production when two pathways are induced; the second pathway, namely MPK1 and c-Fos activation, was dependent on hyphal formation. In this way conidia are tolerated and germination is seen as a danger signal in which the epithelium plays an important initial step to induce a protective inflammatory network (40). *Aspergillus* species are not typical commensals. Lung epithelium not only acts as an efficient barrier, it can also phagocytose *Aspergillus* conidia. Although primary epithelial cells from the respiratory tract phagocytose *Aspergillus* conidia, the killing capacity of these cells is low (41). However, they can control outgrowth, and in addition potent antifungal drugs such as posocanazol can accumulate in the membranes of lung epithelial cells and in this way might contribute to efficient exposure of antifungal drugs during *Aspergillus* infection. Thus epithelial cells can play an important role in fungal infection by restricting fungal outgrowth and initiate potent antifungal host responses.

Monocytes and Macrophages

Residential macrophages are phagocytes that are essential in mediating the first steps of an effective antifungal host defence. Fungi have evolved strategies to escape these initial first steps. *Candida* can inhibit phagolysosome maturation and production of NO

by macrophages, while *Aspergillus* can inhibit phagolysosome acidification (20). In addition, fungi can change the characteristics of macrophages; *Candida* infection can induce a more anti-inflammatory phenotype enhancing fungal survival by switching M1 macrophages to a M2 phenotype (42). Recently, the importance of residential macrophages to control disseminated fungal infection was elegantly demonstrated. A deficiency in accumulation of residential macrophages in the kidney due to a deficiency in the chemokine receptor CX3CR1 led to renal failure and death. Moreover, patients with a polymorphism leading to a decreased function in CX3CR1 were more susceptible to disseminated candidiasis. These data highlight the essential role of residential macrophages, in particular in the kidney to control disseminated fungal infection (43). In addition to macrophages, monocytes can also phagocytose spores and inhibit germination. Monocytes have a potent conidial killing capacity and are essential in the first 48h to control disseminated candidiasis (44). Moreover, monocytes can rapidly differentiate into dendritic cells that transport spores to draining lymph nodes, where they prime and expand *A. fumigatus*-specific T helper cells (45). In addition, monocytes can augment neutrophil conidiacidal activity and play a crucial role in controlling disseminated fungal infection (46).

Neutrophils

Neutrophils are essential to clear fungal invasion, which is underscored by the observation that immunosuppression with prolonged neutropenia is a major risk factor for invasive fungal infections. Various neutropenic mouse models have clearly demonstrated a significant role for neutrophils in disseminated fungal infection. Mice depleted of alveolar macrophages prior to pulmonary aspergillosis recruited neutrophils normally and were able to restrict hyphal tissue invasion, while neutrophil depletion prior to or within 3 h after infection resulted in high susceptibility (47). Neutrophils use oxidative and non-oxidative killing mechanisms, which are both essential in *Candida* and *Aspergillus* infections. The oxidative burst is produced by the enzymes NADPH oxidase and myeloperoxidase. NADPH-oxidase deficiency results in the disease chronic granulomatous disease (CGD), which is associated with an extraordinary increased susceptibility to invasive mold infection, highlighting the importance of NADPH-oxidase in controlling fungal infection. Another specific feature of neutrophils is their capacity to rapidly release granules that mediate extracellular killing. Those granules contain, next to NADPH oxidase and myeloperoxidase other proteins with antimicrobial properties, like lysozymes, lactoferrins, elastases, defensins, gelatinases or cathepsin G (48). In a cell-free supernatant of de-granulated neutrophils of CGD patients a normal amount of iron-free lactoferrin was found, which was capable to decrease fungal growth (49). Additionally, neutrophil elastase and cathepsin B were described to have antifungal activity and elas-

tase was found to contribute to neutrophil extracellular traps (NETs) (50). NETs, which are composed of DNA that forms large fibrillar structures that can bind pathogens and antimicrobial peptides, provide a mechanism to combat hyphae that are too big to be phagocytosed. It was demonstrated that *Candida* can be captured by NETs, however it did not contribute to efficient killing (51). Similarly, NETs were able to limit fungal growth, but not to kill *Aspergillus* conidia and hyphae (50, 52). Bianchi *et al.* have demonstrated that NET formation is deficient in CGD patients and that NET formation can be restored by gene-therapy resulting in successful neutrophil-dependent elimination of *A. nidulans* conidia and hyphae. The neutrophil-associated calprotectin was identified to inhibit growth of *A. nidulans* in the NETs of CGD mice after gene therapy (53). The antifungal activity of calprotectin is not restricted to *Aspergillus*; it was also identified as the main component in NETs controlling *Candida* growth *in vivo* and *in vitro* (54). CGD is not only characterised by a deficiency in neutrophil killing and NET formation. The NADPH oxidase is also present in monocytes and macrophages, and has been shown to be also important for controlling fungal infection (55).

In addition, macrophages and monocytes deficient in NADPH-oxidase have a defect in autophagy, an intracellular mechanism that can control *Aspergillus* outgrowth (56). Whereas autophagy appears to be crucial for *Aspergillus* infection, it seems redundant in candidiasis, although initially it was reported that mice deficient in autophagy were more susceptible to *Candida* infection (57, 58). A selective killing defect by neutrophils for *C. albicans*, but not for *A. fumigatus*, was found in CARD9 deficient neutrophils resulted in abnormal ultrastructural phagolysosomes and massive hyphal outgrowth, which was independent of ROS production and release of azurophilic granules. Neutrophils were found to display two independent killing mechanisms; ROS-dependent that was crucial for clearing opsonized *Candida* and CARD9 dependent that was crucial for unopsonized *Candida* (59). This suggests that neutrophils have an unknown killing mechanism that is specific for *Candida* initiated by CARD9.

IL-17 is a potent mediator of neutrophil recruitment. Next to T helper cells and innate lymphoid cells, neutrophils themselves have been reported as a source for IL-17, in a Dectin-1 and IL-23 dependent manner, and RORγT positive neutrophils exposed to IL-23 and IL-6 express the receptor IL-17RC (60). This suggests an autocrine feedback-loop in recruiting neutrophils to the site of the fungal infection. Mice deficient in IL-17 signalling are more susceptible to systemic candidiasis. However, patients with an IL-17 deficiency suffer from mucosal candidiasis, but do not develop invasive candidiasis, and are not more susceptible to invasive mould infection. It is still an open question to what extend IL-17 determines neutrophil faith and function at the mucosa. In a mouse model of oropharyngeal candidiasis IL-17RA-/-and IL-23-/- mice depleted for neutrophils

showed higher fungal burden compared to non-neutropenic animals, suggesting that IL-17 deficiency does not result in a complete neutrophil function at the site of infection (61). In addition, the recruitment of neutrophils by vaginal epithelial cells producing S100 alarmins was independent of IL-17 production (62).

Dendritic cells

Dendritic cells (DCs) achieve a crucial patrolling function. They are located in the mucosa and migrate to the draining lymph nodes to activate T cells. DCs stimulated with the fungal pathogens *Aspergillus* and *Candida* showed increased activation markers like CD80 and CD86, as well as IL-8 and IL-12p70 production (63). Moreover, DCs played a crucial role for the host response against *Candida* via the production of type-I IFNβ in a Dectin/Syk/IRF5 dependent pathway (64-66). DCs are mainly involved in activation and shaping T helper (Th) responses. During pulmonary *Aspergillus* infection conidial transport from the lung to the lung draining lymph nodes, priming of CD4+ T cell responses and the prevention of the pulmonary fungal burden was mediated by monocyte derived CD11b+ DC population in a murine model (45). *Aspergillus* conidia triggered a protective Th1 and Treg responses, whereas *Aspergillus* hyphae triggered an anti-inflammatory Th2/Th17 profile (67). The protective DC responses were dependent on the PI3K/Akt/mTOR pathway, while the anti-inflammatory DCs depended on STAT3/IDO activation (67).

The direction of the airway inflammation in mice into either Th2 or Th17 was dependent on TNFα produced by monocyte-derived DCs: BALB/c mice showed a high amount of TNFα-producing DCs promoting Th17 responses with subsequent neutrophils recruitment, while C57BL/6 mice displayed a low amount of TNFα producing DCs, associated with high IL-5 levels and eosinophilia (68). Plasmacytoid DCs (pDCs) represent a specific subset of DCs, and in the context of fungal infections have not been studied in great extend. In a mouse model of invasive aspergillosis a specific influx of pDCs was observed, and depletion of pDCs resulted in an increased susceptibility to invasive aspergillosis. *In vitro* analysis showed that hyphal growth was inhibited by pDCs in a contact-independent manner. Interestingly, pDCs produced inflammatory cytokines, such as IFNα and TNFα that were independent of TLR9 (69). In contrast, pDCs in a vaginal candidiasis model were suggested to mediate tolerance, since pDCs that were were present in the draining lymph nodes of the infection lacked the upregulation major histocompatibility complex class II, CD80, CD86, and CD40 (70). This could points to a crucial difference in pDC responses induced by *Aspergillus* or *Candida* that might be dependent on the site of infection.

Innate lymphoid cells

Recently, a novel population of innate lymphocytes called innate lymphoid cells (ILCs) has been identified. ILCs are analogous to Th helper subsets, but differ because they lack the T cell receptor. They have been named according to their cytokine profile. ILC1 cells express T-bet and produce IFNγ, ILC2 cells express GATA3 and produce IL-5 and IL-13, and ILC3 cells express RORγt and produce IL-17 and IL-22. Only a few studies so far have deciphered a role for innate lymphoid cells in the host defence against *Aspergillus* or *Candida*. The IL-17 producing ILC3 cells have been described as being important in the defence against and the control of pathogens at the mucosal barrier. *C. albicans* is a potent inducer of IL-17 (71) and mice lacking IL-17 are highly susceptible to *Candida* infections (72). As described earlier, patients with an IL-17 deficient phenotype such as hyper IgE syndrome (73) and patients with chronic mucocutaneous candidiasis (74), suffered from mucosal candidiasis, but the lack of IL-17 in these patients has been predominantly attributed to Th17 cells. Innate lymphoid cells would supply a faster source of IL-17 than the adaptive Th17 cells, and they represent an additional important source of IL-17.

The first clues that ILCs play an important role in controlling fungal infection and colonization come from experimental mouse models. Fungal control in a murine model of oral candidiasis was mediated by IL-17 secreting ILCs and both, ILC depleted RAG-/- and RORC+ ILC-/- mice completely failed to control fungal outgrowth (75). The relevance of ILCs in mediating pulmonary host responses in the lung was demonstrated in a study where *Candida* airway exposure was able to protect against *Pseudomonas aeruginosa*-induced lung injury, an effect that was dependent on pulmonary ILCs that expressed IL-22 (76). It has been demonstrated that ILCs play a role in the pathogenesis of asthma and allergic reactions, and the contribution of GATA3+ ILCs to allergic reactions mediated by *Aspergillus* has recently been studied in patients with chronic sinusitis. These patients showed a significant proliferation of GATA3+ ILCs and increased GATA3+ ILCs responsiveness to IL-33 induced by *Aspergillus* extract which resulted in an increased IL-13 production (77). Interestingly, also invariant NKT (iNKT) cells were found to contribute to airway hyperactivity in an IL-33 dependent manner, directly activated by the *Aspergillus* glycosphingolipid Asperamide B (78). This might explain one mechanism via which *Aspergillus* can directly induce allergic responses in an immunocompetent host, and suggests a critical role for ILCs in fungal-induced allergic reactions. The role of ILCs in an immunosuppressive status during invasive fungal infection still needs to be investigated.

Natural Killer Cells

Natural Killer (NK) cells contribute to the rapid innate immune response against invading pathogens in an antigen-independent manner, mainly by recognizing infected cells

and induce cell death of the infected cell. NK cells also contribute to the host defence against fungal infections. The additional depletion of NK cells in a T/B-cell-deficient SCID mice increased the susceptibility to systemic candidiasis, while it had no (or even an opposite) effect in immunocompetent mice (79). NK cells might substitute for specific functions of B and T cells when these cells are absent, which is in line with the observation that NK cell activation by *Candida* results in the production of high amounts of GM-CSF, IFNγ and TNFα; cytokines that are also produced by T cells. Although phagocytosis by NK cells did not inhibit hyphal growth of *Candida*, antifungal activity against *Candida* was observed that was mediated by extracellular perforin production by NK cells (80). Comparably, NK cells could kill *Aspergillus* hyphae via a perforin-mediated mechanism, while *Aspergillus* conidia were not affected (81). Bouzani *et al.* also showed that only germinated morphological forms of *Aspergillus* were able to stimulate NK cells to produce high amount of IFNγ. However, in contrast to Voigt *et al*. and Schmidt *et al*., they propose a perforin-independent killing mechanism, since they observed that the killing was mediated via a soluble factor (82). NK cells are potent producers of IFNγ, and IFNγ is crucial for controlling *Aspergillus* infection, which is highlighted by the fact that IFNγ knock-out mice displayed an increased susceptibility to invasive aspergillosis. In a murine model of invasive aspergillosis, in which neutrophils were depleted, NK cells were the major population of cells that were able to secrete IFNγ and to control fungal growth in the lung during early infection. The depletion of NK cells in IFNγ-deficient mice did not result in increased severity of the infection, while depletion of NK cells in wild type mice resulted in a similar increases susceptibility to infection that was observed in IFNγ-deficient mice. These data strongly suggest that in neutropenic conditions the protective IFNγ responses during early invasive aspergillosis are critically dependent on NK cells. (83).

Platelets

Classically, platelets, mediate blood coagulation after interruption of the endothelium. In addition, platelets are capable to mediate innate immune functions, especially during bloodstream infections. Although *in vitro* studies failed to show the capacity of *C. albicans* to aggregate human platelets, *in vivo* platelets bound to *C. albicans* and were activated after bloodstream injection in a murine model (84). Platelets produced peptides like Rantes or platelet factor 4 with antimicrobial activity against *Candida* and platelet-rich plasma inhibited the growth of *Candida* (85). In *Aspergillus* infection, the production of the proinflammatory IL-8 from human monocytes is enhanced by platelets activated by *Aspergillus* hyphae, and all *Aspergillus* morphotypes had the capacity to activate platelets (86). This seems to be an interesting feedback-loop, since *Aspergillus* germination

and hyphal formation was inhibited by activated platelets (87). Interestingly, not only the direct contact of the platelets to *Aspergillus* was necessary, also *Aspergillus* cell culture supernatant was able to activate platelets, suggesting a potentially harmful contribution to systemic infection by increasing thrombotic status during invasive aspergillosis (87).

CONCLUSIONS AND FUTURE PERSPECTIVES

During past years fungal pathogens have been of rising interest especially due to the increasing number of immunocompromised patients. In order to combat fungal infections, the human immune system needs to be able to efficiently sense the fungal pathogen, and to activate a plethora of immune cells that are able to engulf and clear the pathogen. In this regard PRRs are of significant importance. Numerous studies revealed beneficial as well as detrimental roles of different TLRs in fungal infections. Furthermore, several studies on murine models are contradictory, probably due to the differences in the experimental setup, mouse strains that are used, as well as differences in fungal strains. Although many PRRs that recognize fungal PAMPs have been identified, there is still a large amount of unexplored PRRs that could contribute to antifungal host defence. Moreover, the interactions that have been described between PRRs point to an even more complex dynamic cell wall recognition system on immune cells. The knowledge that there could be many fungal PRRs that are dynamically expressed and have the capacity to interact with each other indicates that understanding fungal recognition at the host cell membrane needs further studies and will be challenging and complex.

Fungal recognition as well as the interplay between the innate immune cells forms the initial inflammatory network that is needed to control fungal infection. A better understanding of the role of epithelial cells in fungal infection has led to an increased awareness that non-classical immune cells, such as epithelial cells, endothelial cells and platelets, also play a crucial role in the orchestration of an efficient antifungal host response. In addition, new subsets of immune cells, such as innate lymphoid cells have come to stage and NK cells have gained interest in their contribution to control fungal infection. These new players next to the traditional and well-known professional phagocytes such as neutrophils and monocyte/macrophages open up relevant new questions. To what extend does immunosuppression with corticosteroids affect these new players? And in turn does this influence the function of the classical phagocytes? Does NADPH-oxidase deficiency influence effective epithelial and endothelial antifungal host responses? In addition, recent studies have suggested an innate immune memory in NK cells as well as in monocytes, which opens new horizons regarding the host-fungus interactions. Hence, not only CLRs, but also some TLRs were shown to mediate immune

responses to a microbe, upon a second encounter, by either strengthening or diminishing host responses by the means of host chromatin remodelling that can shape the host immune response (88, 89), making these receptors interesting candidates for further investigation as potential targets for vaccines.

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CHAPTER 3

Aspergillus cell wall chitin induces anti- and proinflammatory cytokines in human PBMCs via the Fc-γ Receptor/Syk/PI3K pathway

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ABSTRACT

Chitin is an important cell wall component of *Aspergillus fumigatus* conidia of which hundreds are inhaled on a daily basis. Previous studies have shown that chitin has both anti- and pro-inflammatory properties; however the exact mechanisms determining the inflammatory signature of chitin are poorly understood, especially in human immune cells.

Human peripheral blood mononuclear cells were isolated from healthy volunteers and stimulated with chitin from *Aspergillus fumigatus*. Transcription and cytokine production of the pro-inflammatory IL-1β and the anti-inflammatory IL-1Ra were measured by qPCR or ELISA from the cell culture supernatant, respectively. Chitin induced an anti-inflammatory signature characterized by the production of IL-1Ra in the presence of human serum, which was abrogated in immunoglobulin depleted serum. Fcγ-receptor-dependent recognition and phagocytosis of IgG-opsonized chitin was identified as a novel IL-1Ra-inducing mechanism by chitin. IL-1Ra production induced by chitin was dependent on Syk kinase and PI3K activation. In contrast, co-stimulation of chitin with the PRR ligands lipopolysaccharide, Pam3Cys or muramyl dipeptide, but not β-glucan, had synergistic effects on the induction of pro-inflammatory cytokines by human PBMCs. In conclusion, chitin can have both pro- and anti-inflammatory properties, depending on the presence of pathogen associated molecular patterns and immunoglobulins, thus explaining the various inflammatory signatures reported for chitin.

IMPORTANCE

Invasive and allergic aspergillosis are increasing health care problems. Patients get infected by inhalation of the airborne spores of *Aspergillus fumigatus*. A profound knowledge of how *Aspergillus* and its cell wall components are recognized by the host cell and which type of immune response it induces is necessary to develop target-specific treatment options with less severe side-effects compared to treatment options to date. There is controversy in the literature about the receptor for chitin in human cells. We identified the Fcγ-receptor and Syk/PI3K-pathway via which chitin can induce anti-inflammatory immune responses by inducing IL-1 receptor antagonist in the presence of human immunoglobulins, but also pro-inflammatory responses in the presence of bacterial components. This explains why *Aspergillus* does not induce strong inflammation just by inhalation and rather fulfills an immune-dampening function. While in a lung co-infected with bacteria, *Aspergillus* augments immune responses by shifting towards a pro-inflammatory reaction.

INTRODUCTION

Conidia of the opportunistic fungus *Aspergillus fumigatus* are responsible for allergic syndromes, especially allergic bronchopulmonary aspergillosis. This occurs especially in patients with asthma or cystic fibrosis, who are chronically colonized with *Aspergillus* (1). Several *Aspergillus* proteins are known as allergens, driving pathology in allergic bronchopulmonary aspergillosis. In addition to proteins, the polysaccharide chitin is also known to induce allergy by causing accumulation of interleukin (IL)-4 expressing innate immune cells (2). Humans do not produce chitin, but express proteins that can degrade or bind chitin, such as chitinases and chitinase-like proteins, respectively (3). Polymorphisms in chitinase genes (4) or elevated serum levels of chitinase-like proteins have been associated with asthma (5), although the exact mechanism describing how chitinases and chitin interact with immune cells and trigger immune responses is not yet completely understood.

Chitin is one of the major fibrillar components of the *Aspergillus* cell wall, and is covalently bound to β-(1,3)-glucans (6). While in a resting conidium polysaccharides are shielded by a rodlet and melanin layer, during germination chitin is exposed on the mycelium surface and can interact with cells of the innate immune system (6). *Aspergillus* cell wall polysaccharides serve as pathogen associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) to induce an innate immune response with consequent induction and shaping of an adaptive immune response (7). While β-(1,3)-glucan is known as the ligand for dectin-1 (8), recent studies

propose different identities of the chitin recognition receptor and the induced immune response. Schlosser *et al.* identified FIBCD1 as a high-affinity chitin binding receptor of the intestine that controls immune responses against ingested parasites and fungi (9). Additionally, chitin was described to have pro-inflammatory properties by inducing IL-17 via the Toll-like receptor (TLR)2 pathway (10) in murine cells, and a recent study suggested recognition of chitin via the mannose receptor (MR) on the surface of the phagocytes and by the nucleotide-binding oligomerization domain (NOD)2 and TLR9 receptors in the cytoplasm, leading to the induction of the anti-inflammatory cytokine IL-10 in mouse macrophages (11). No pattern recognition receptor or signalling pathway triggered by chitin has been identified in human immune cells to date. In the present study, we aimed to elucidate the immunological properties of chitin isolated from the *A. fumigatus* cell wall and its receptor in human peripheral blood mononuclear cells, and the mechanism and circumstances under which chitin can switch between anti-inflammatory immune responses towards pro-inflammatory responses.

METHODS:

Volunteers and patients

Blood was collected from healthy volunteers or patients by venous blood puncture. Two NOD2-deficient individuals had the homozygous NOD2 insertion of a cysteine at position 1007 (rs2066847) that results in a frameshift of the coding sequence, which was analysed and described previously (12). Serum was used from two patients with a deficiency in Mannose binding lectin (MBL) with serum MBL levels below 0.04 mg/L. One patient had the autosomal recessive mutation in *FERMT3*, the gene encoding kindlin-3 leading to the leukocyte adhesion deficiency type III (13, 14).

Ethics statement

All experiments were performed and conducted in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethical Committee (nr.2010/104). Blood from volunteers and patients was taken after written informed consent was obtained.

PRR ligands, blockers and other stimuli

E.coli lipopolysaccharide (LPS) (1 ng/mL) (TLR4 ligand, *E. coli* serotype O55:B5, Sigma-Aldrich St. Louis, MO USA), Pam3Cys (1 µg/mL) (TLR2 ligand, EMC microcollections, Tübingen, Germany), N-acetylmuramyl-ananyl-D-isoglutamine (MDP) (5 µg/mL) (NOD2

ligand, Sigma-Aldrich). Fungal cell wall β-(1,3)-glucan (10 µg/mL) (dectin-1 ligand) was kindly provided by Prof. David L Williams (East Tennessee State University, Johnson City, TN, USA). This polysaccharide was isolated from *C. albicans* yeast and it was verified it was exempt of proteins. The suspension contained water-insoluble microparticles (1-5 µm) (15).

The dectin-1 inhibitor GE2 was a kind gift of Prof. Gordon Brown (University of Aberdeen, Scotland). *Bartonella quintana* LPS was prepared and purified as described elsewhere (16) and used as a TLR4 inhibitor (100 ng/mL) (17); isotype control mouse IgG1 (10 µg/mL) (eBioscience, Halle-Zoersel, Belgium); anti-TLR2 (10 µg/mL) (eBioscience); isotype control goat IgG (10 µg/ml) (R&D Systems Minneapolis, MN, USA); isotype control mouse IgG1κ (10 µg/mL) (Biolegend, San Diego, CA, USA); anti-human mannose receptor (MR) (anti-human CD206, 10 µg/mL) (Biolegend); isotype control goat IgG (10 µg/ml) (R&D Systems Minneapolis, MN, USA); anti-human β_2-integrin (anti-CR3) (10 µg/ml) (R&D Systems); isotype control mouse IgG2b (10 µg/mL) (R&D Systems Minneapolis, MN, USA); anti-human CD32 (anti-Fcγ Receptor II) (10 µg/mL) (Stemcell Technologies SARL, Grenoble, France); Wortmannin (100ng/mL, dissolved in DMSO) (Sigma), R406 Syk-kinase inhibitor (5 µM, dissolved in DMSO) (Invivogen, Toulouse, France); cytochalasin D (10 µg/mL, dissolved in DMSO) (Sigma-Aldrich); polymyxin B (2 µg/mL) (Sigma-Aldrich), 2-hours pre-incubation with chitin at 37˚C; Human Immunoglobulins for intravenous administration (IVIG) (Nanogam, Sanquin, Amsterdam, The Netherlands) was dialysed in PBS before use. Mannose binding lectin (MBL) was purified from human plasma, as described in a previous study (18).

Purification and characterization of chitin from the A. fumigatus cell wall

Chitin was isolated from the *A. fumigatus* cell wall (*A. fumigatus* CEA17_Δ*akuB*^{KU80} strain (19); mycelia collected after 20 h growth at 37˚C in the liquid Sabouraud medium) according to the method described earlier (20, 21). However, the resultant chitin showed ~3% β-(1,3)-glucan contamination when checked by gas chromatographic analyses (22). This contamination which could have important immunological consequences, was cleared upon recombinant endo-β-(1,3)-glucanase treatment. In brief, chitin preparation (5 mg in 0.5 mL of 50 mM acetate buffer, pH 6.0) was treated with endo-β-(1,3)-glucanase (20 µL containing 5 µg protein) at 37˚C for overnight followed by centrifugation and checking the supernatant for reducing sugar released by *p*-aminobenzoic acid assay using 4-hydroxy-benzhydrazide (23). This was repeated till no more reducing sugar was released into the supernatant. Further, the chitin preparation was washed thoroughly with sterile water and the absence of β-(1,3)-glucan was confirmed by gas chromatographic analysis. The degree of acetylation in the chitin preparation was determined

by three different methods: the Cibacron brilliant red 3B-A dye binding method (24), a UV spectrophotometry method using dual standards [glucosamine (GlcN) hydrochloride and *N*-acetyl-glucosamine (GlcNAc)] For UV analysis, the chitin was solubilized upon ultra-sonication. A stock aqueous chitin suspension (1 mg/mL) was prepared by ultra-sonication using a probe-sonicator (Soniprep 150, MSE, London, UK). The sonicator was set to 50% power and 50% tune reaching 16 amplitude microns for 30 seconds. The sonication reduced the molecular weight while not affecting the degree of acetylation (25, 26) and a Fourier Transform Infrared Spectroscopy (FT-IR) method using the absorption ratio A_{1655}/A_{3450} (27, 28)

For FT-IR, dry chitin was suspended uniformly between IR window and spectrum was recorded (1400-4000 cm^{-1}) in a JASCO FT/IR-6100 apparatus, subtracting the spectra of air.

Experiments were performed with four different batches of chitin purified from the *A. fumigatus* mycelial cell wall with a final concentration of 10 µg/mL.

To determine the size of the chitin particles, chitin suspension was subjected to Flow cytometry (FC500 Flow cytometer, Beckman Coulter) and compared with reference beads of 0.1 µm, 0.25 µm (Thermo Scientific), 3, 6 and 10 µm (Flow-Check Pro Fluorospheres, Beckman Coulter). The data were analyzed using Kaluza (Analysis Version 1.3, Beckman Coulter).

PBMCs isolation

Venous blood was drawn in 10 mL EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI+, consisting of RPMI-1640 culture medium (Dutch modification, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the concentration was adjusted to $1x10^7$ cells/mL.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning, NY, USA) at a final concentration of $2.5x10^6$/mL in an end volume of 200 µL per well. Stimulations were performed in the presence of 10% human serum, either not depleted or depleted for all immunoglobulins (BBI solutions, Cardiff, UK). Serum was either complement active, if not otherwise indicated, or heat-inactivated by incubation for 30 minutes at 56°C in a water bath according to a commonly used protocol (29). After 1 hour pre-incubation with inhibitor or medium,

stimuli or medium were added. Cells were incubated at 37˚C with 5% CO_2, after 24 hours, supernatants were collected and stored at -20˚C.

Cytokine measurements

IL-1β, tumor necrosis factor (TNF)α, IL-6, IL-8, IL-10 and IL-1Ra were measured in the cell culture supernatants using commercial ELISA kits (IL-1β, TNFα and IL-1Ra: R&D Systems; IL-6, IL-8 and IL-10: Sanquin) according to the instructions supplied by the manufacturer.

Pulldown assay of chitin-binding immunoglobulins in human serum

To deplete serum from chitin-binding immunoglobulins, 1 mL of RPMI supplemented with 10% serum containing either 1 mg chitin from a stock concentration of 10 mg/mL or the equal amount of distilled water (for mock treatment) was incubated overnight at 37˚C and shaken at 130 rpm. On the next day, the chitin containing or mock treated suspension was centrifuged (20 minutes, 14000 rpm, room temperature) and the supernatant without beads was recovered. The depletion efficacy was checked by a chitin-binding IgG ELISA.

Chitin-binding IgG ELISA

A polystyrene 96-well plate was coated with 10 µg/mL chitin (100 µL per well) overnight at room temperature. After washing and blocking with PBS with 1% bovine serum albumin (BSA) for 1 hour, wells were incubated with 10% serum in RPMI+, mock treated serum and depleted serum for 1 hour. IgGs bound to chitin were detected by using an anti-human IgG (whole particle) labelled with peroxidase in a concentration of 1:1000 (Sigma-Aldrich). Enzymatic reaction was started by adding the TMB and H_2O_2 containing substrate, which was stopped after 10 minutes by 10% sulphuric acid solution. The optic density was measured by photometric ELISA reader.

Quantitative PCR (qPCR)

RNA was isolated from $1x10^6$ PBMCs after stimulation for 4 hours and 24 hours with *Aspergillus* chitin either in the presence of medium or 10% human serum using Trizol reagent (Invitrogen) according to a protocol supplied by the manufacturer. RNA (500 ng) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Hercules, Bio-Rad Laboratories, CA). Quantitative PCR (qPCR) analysis was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 real-time PCR system. As PCR protocol the following conditions were used: 2 minutes 50˚C, 10 minutes 95˚C followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 minute. For the amplification of hIL-1Ra the primers 5'- GCCTCCGCAGTCACCTAAT-3'

and 5'- TCCCAGATTCTGAAGGCTTG-3' and for the amplification of hIL-1β the primers 5'- GCAACTGTTCCTGAACTCAACT-3' and 5'- ATCTTTTGGGGTCCGTCAACT-3' were used. To correct for differences in loading concentrations of RNA between the different conditions, qPCR results were corrected with the housekeeping gene β2 microglobulin (β2m) amplified using the primers 5'- ATGAGTATGCCTGCCGTGTG-3' and 5'-CCAAATGC-GGCATCTTCAAAC-3'. Primer efficacy was evaluated using a standard curve. IL-1Ra Ct values were compared with the β2m Ct by calculating the delta Ct and the fold-change was calculated relative to the RPMI-stimulated to determine the effect of stimulation with chitin on IL-1Ra expression.

Statistical analysis

The Wilcoxon signed rank test was used to determine differences between stimulation with and without inhibitors or between different sera. A p-value of < 0.05 was considered statistically significant ($^{*}=p < 0.05$, $^{**} = p < 0.01$ and $^{***} = p < 0.001$). Graphs represent cumulative results of all performed experiments and are presented as mean ± standard error of the mean (SEM). Data were analyzed with GraphPad Prism v 5.0.

RESULTS

Aspergillus fumigatus cell wall chitin produces IL-1Ra in the presence of serum

The chitin samples purified from *A. fumigatus* cell wall contained 70% of the particles showing sizes less than 0.5 μm (Fig 1; and a degree of acetylation of ~91% (Supplemental Figure 1). Purity of the chitin was ensured: first, the 3% of β-(1,3)-glucan still remaining bound to chitin after the final chemical extraction was removed by an endo-β-(1,3)-glucanase treatment (see materials and methods section); second, the absence of endotoxin contaminations were ensured by the pre-treatment of chitin suspension with polymyxin B. In order to identify immunological function of chitin, human PBMCs were stimulated *in vitro* with chitin in the presence or absence of human serum. The pro-inflammatory cytokines IL-1β, TNFα, IL-6 (Fig 2A) were not detectable in the cell culture supernatant by ELISA in presence or absence of chitin. In addition, the anti-inflammatory IL-10 was not induced by chitin, neither on protein level (Fig 2A) nor on transcription level, while LPS and LPS with chitin induced high transcription of IL-10 (Fig 2B). Although IL-8 was high in presence of chitin and serum, serum without chitin also induced a high level of IL-8, indicating that this high level of IL-8 is not due to the presence of chitin (Fig 2C). However, chitin induced significant amounts of IL-1Ra compared to the medium control (Fig 3A), in a dose-dependent manner (Fig 3B). A kinetic analysis of IL-1Ra mRNA

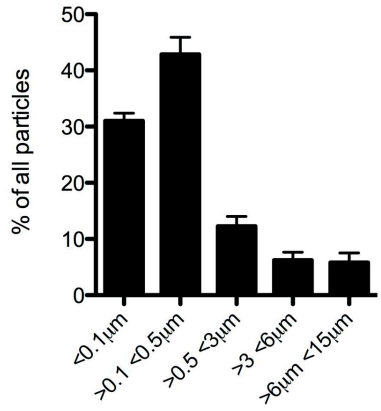

Figure 1: *Characterisation of chitin particles purified from Aspergillus mycelium*

Chitin particles were measured by flow cytometry and compared with reference beads; the distribution of chitin particles was calculated. Mean and standard deviation of 5 independent measurements of three different batches of *Aspergillus* chitin are depicted.

expression showed that IL-1Ra transcription peaked after 4 hours of exposition of cells to chitin (Fig 3C). The absence of transcription of IL-1β confirmed the lack of detection of this cytokine in the culture supernatant (Fig 2A). In conclusion, serum was required for the induction of IL-1Ra transcription and release by PBMCs upon chitin stimulation.

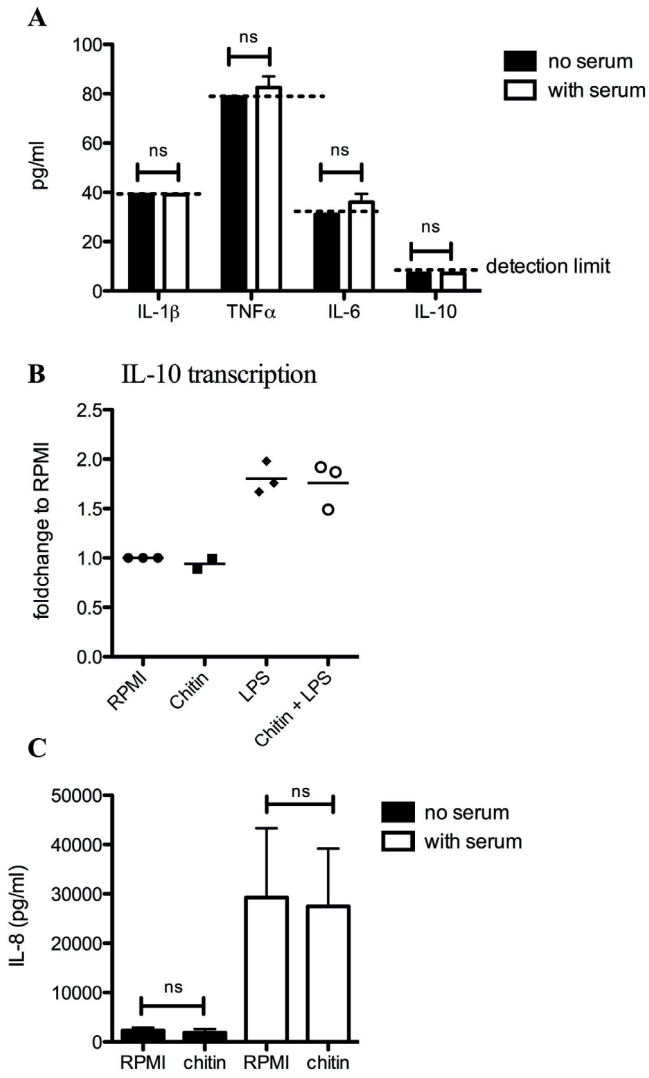

Figure 2: *Aspergillus* chitin *does not induce pro-inflammatory cytokines or the anti-inflammatory cytokine IL-10*

(A) IL-1β, TNFα, IL-6, IL-10 (n=6) and (C) IL-8 – production (n=8), and (B) IL-10 transcription (n=3) , were measured (A+C) either in the cell culture supernatant, or (B) in mRNA, of PBMCs of healthy controls that were stimulated (A+C) with *Aspergillus* chitin either the presence of medium (black bars) or human serum (withe bars) or (B) with *Aspergillus* chitin, LPS and LPS with *Aspergillus* chitin.

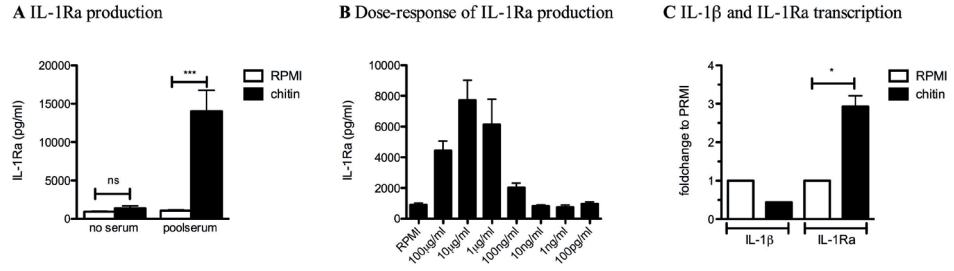

Figure 3: *Chitin induces IL-1Ra-release and transcription of IL-1Ra, but not IL-1β in the presence of human serum*

(A) IL-1Ra production measured in the cell culture supernatant of PBMCs of healthy controls after 24 hours-stimulation either in the presence of RPMI (white bars) or human serum (black bars) (A) with 10 µg/ml *Aspergillus* chitin (n=9) or (B) with the different dosages 1,10 and 100 µg/ml *Aspergillus* chitin in the presence of human serum (n=3). (C) IL-1β and IL-1Ra transcription was measured by qPCR after 4-hours stimulation in the presence of human serum (n = 3). Statistical analysis was performed using a (A) Wilcoxon signed rank test or (C) paired t test (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Dectin-1, TLR2, TLR4, MR and NOD2 are not involved in chitin-induced IL-1Ra induction

Subsequently, we aimed to identify the receptor that recognizes chitin that mediates IL-1Ra production by stimulation of PBMCs with chitin. Blocking dectin-1 with GE2 antibody did not affect the chitin-induced IL-1Ra production (Fig 4A). In addition, blocking TLR2 or TLR4 also did not influence the IL-1Ra production by chitin (Fig 4B, C). Since MR and NOD2 have been described as recognition receptors for *Candida albicans* derived chitin (11), we stimulated PBMCs with chitin and human serum in the presence of a MR neutralizing antibody as well as stimulated PBMC of NOD2 deficient patients with chitin and the NOD2 ligand MDP. Blocking MR did not result in significant differences of the production of IL-1Ra (Fig 4D), and as depicted in Fig 4E, MDP-stimulation did not result in any IL-1Ra production in the NOD2 deficient patients, while chitin induced IL-1Ra in equal amounts as in the healthy control. In conclusion, chitin-induced IL-1Ra is Dectin-1-, TLR2- and 4-, MR- and NOD2-independent.

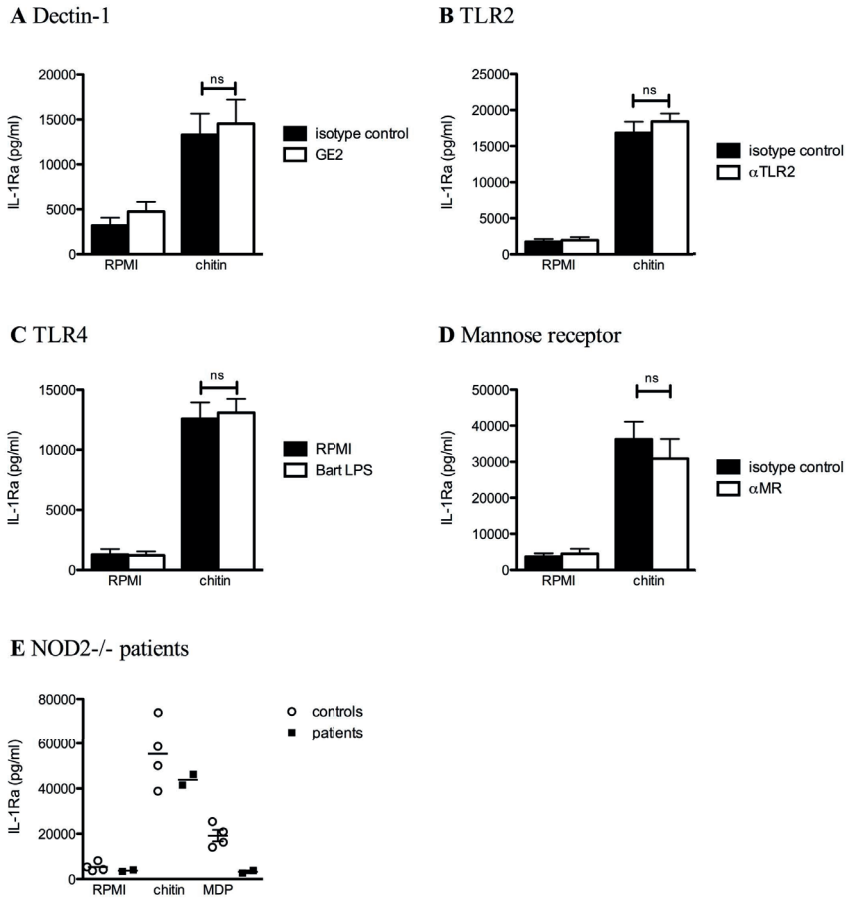

Figure 4: *Dectin-1, TLR2, TLR4, MR and NOD2 are not involved in the chitin-induced IL-1Ra*

Chitin-induced IL-1Ra in culture supernatants of PBMCs of healthy volunteers stimulated in the absence or presence of a neutralizing antibody or molecule, (A) like GE2 for blocking dectin-1 (n = 5), (B) αTLR2 for blocking TLR2 (n=6) and (C) Bartonella LPS for blocking TLR4 (n=8), and (D) αMR for blocking the mannose receptor (n=12), (E) or of two NOD2 deficient patients. IL-1Ra was measured in the cell culture supernatant by ELISA. Statistical analysis was performed with the Wilcoxon Signed Rank test (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Immunoglobulin opsonisation mediates chitin-induced IL-1Ra

Since none of the PRRs known to recognize fungi was identified as the receptor for the recognition of chitin to induce IL-1Ra while human serum was crucial, we engaged additional experiments to decipher the role of different components present in human serum. In the presence of heat-inactivated serum IL-1Ra was still induced, although

there was a lower induction of IL-1Ra compared to non-heat-inactivated serum (Fig 5A). Since MBL is an important polysaccharide-binding component, we stimulated PBMCs of healthy controls with chitin in the presence of MBL deficient serum. However, IL-1Ra was still induced by sera exempt of MBL (Fig 5B). CR3 is a known receptor recognizing complement-opsonized particles (30) and β-glucan (31, 32). IL-1Ra production was not abolished and even significantly increased when CR3 was blocked (Fig 5C). To further validate this finding and avoid a masking effect of the blocking antibodies inducing IL-1Ra in combination with chitin, PBMCs isolated from a Kindlin-3 (which is crucial for maximal CR3 signalling (33))-deficient patient were stimulated with chitin. Comparable to CR3 blocking, Kindlin-3 deficiency did not result in diminished IL-1Ra levels (Fig 5D).

In contrast to the partial effects observed with heat-inactivation of the serum, the induction of IL-1Ra by chitin was completely abolished when serum was depleted of all immunoglobulins (Fig 5E). This result suggested that IgGs are responsible for the IL-1Ra induction by chitin. To demonstrate this, human immunoglobulins (IVIGs) were added to the immunoglobulin free serum, wherein IL-1Ra production could be restored in a dose-dependent manner, indicating that immunoglobulins are required for the induction of IL-1Ra by chitin (Fig 5F). Fcγ receptors recognize the Fc part of IgG and thus recognize IgG opsonized pathogens and particles. An Fcγ Receptor II (FcγRII) neutralizing antibody significantly reduced chitin-induced IL-1Ra production (Fig 5G). To further elucidate whether chitin was directly bound and opsonized by IgG, we incubated 10% diluted serum or 1 mg/ml IVIGs in a polystyrene plate coated with chitin and subsequently detected bound IgG with an anti-Fc-antibody. A significant amount of IgGs bound to chitin in both samples (Fig 5H). To further elucidate whether the IL-1Ra induction was caused by immunoglobulins binding specifically to chitin, human serum was depleted from chitin-binding immunoglobulins. The efficacy of the pull-down experiment was tested by ELISA resulting in a reduction of 73,5 % of chitin-binding antibodies (Fig 5I). Stimulation of PBMCs with chitin in the presence of serum depleted of chitin-binding IgGs resulted in a similar significantly lower IL-1Ra induction (Fig 5J).

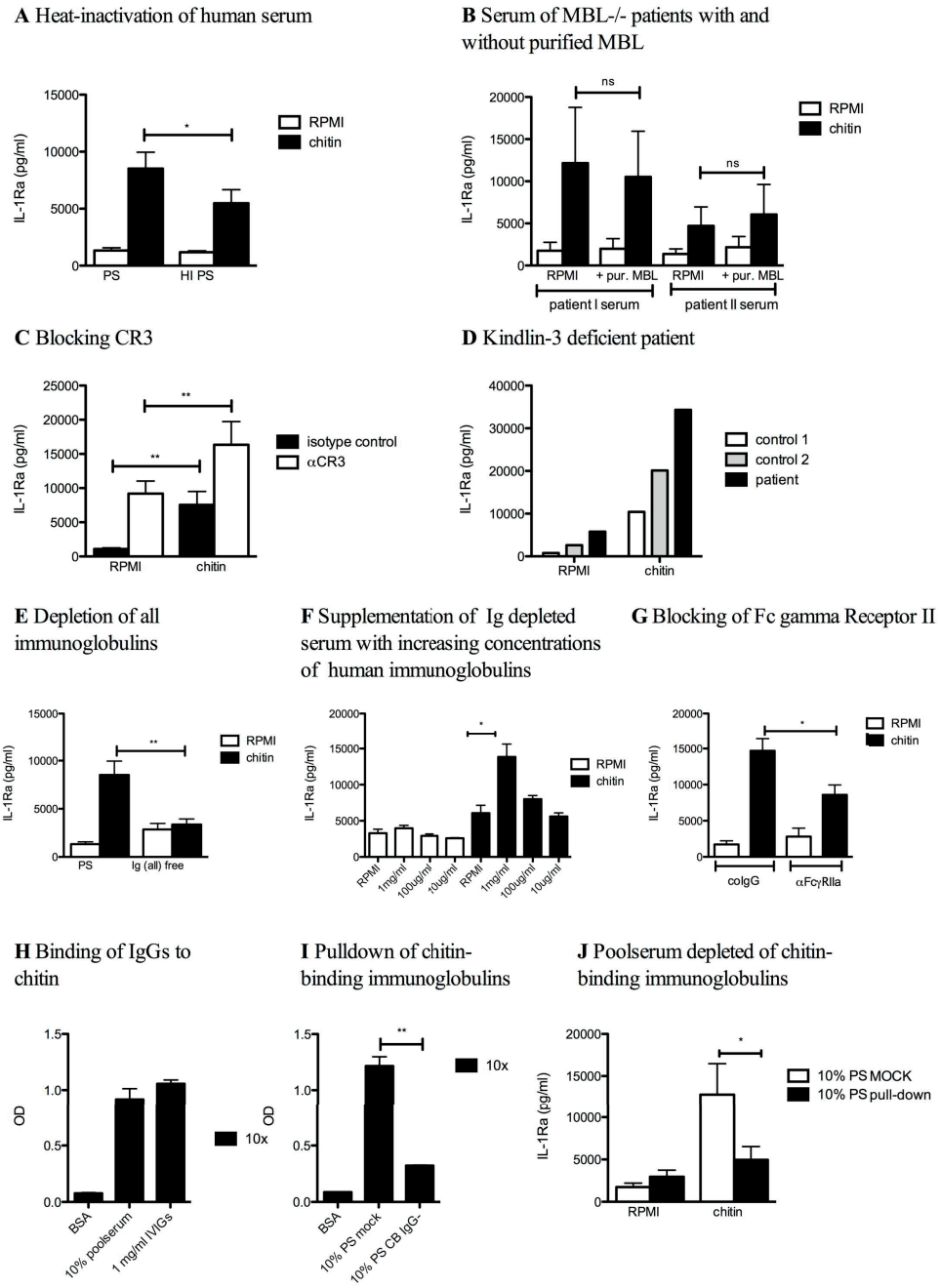

Figure 5: *Complement and immunoglobulin opsonisation mediate the chitin induced IL-1Ra*

PBMCs of healthy volunteers were stimulated with RPMI or chitin in the presence of (A) poolserum (PS) or heat-inactivated poolserum (HI PS) (n=11) (B) MBL deficient serum with and without additional purified MBL (n=3), (C) isotype control or CR3 neutralizing antibody (n= 8). (D) PBMCs of a Kindlin-3 deficient patient or two controls were stimulated with chitin. (E) PBMCs of healthy volunteers were stimulated with RPMI or chitin in the presence of poolserum or immunoglobulin depleted serum (Ig-) (n = 11), or (F) Ig depleted serum supplemented with decreasing concentrations of human immunoglobulins (IVIGs) (1mg/mL, 100µg/mL, 10µg/mL) (n = 6). (H+I) Binding of IVIGs to chitin was determined in by coating an ELISA plate with chitin and a secondary incubation with (H) 10% poolserum or 1 mg/ml IVIGs or (I) 10% poolserum after mock treatment or after pull-down of chitin-binding immunoglobulins and the OD was measured (n= 6). (G+J) PBMCs of healthy volunteers were stimulated with RPMI or with chitin in the presence of (G) an anti- FcγRII antibody or the isotype control (n=3), or (J) in the presence of mock treated poolserum or with poolserum depleted from chitin-binding immunoglobulins. (A-F, G and J) IL-1Ra was measured in the cell culture supernatant by ELISA. Statistical analysis was performed with the Wilcoxon Signed Rank test (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Chitin-induced IL-1Ra is dependent on actin polymerisation and signals via Spleen tyrosine kinase and PI3Kinase

Having identified opsonisation with IgG as the main mechanism for the IL-1Ra induction by chitin, we further deciphered whether phagocytosis and the down-stream signalling of Fcγ-receptor are needed for the induction of IL-1Ra by chitin. We stimulated PBMCs with chitin and human serum and blocked either the actin polymerisation with cytochalasin D (Fig 6A) or the downstream-signalling kinases of the Fcγ-receptor spleen tyrosine kinase (Syk) (Fig 6B) or PI3Kinase (Fig 6C) or the combination of Syk and PI3Kinase. All resulted in a significant reduction of IL-1Ra, while IL-1Ra was completely abolished when Syk and PI3K were blocked simultaneously (Fig 6D). Taken together these findings indicate that recognition and phagocytosis via the Fcγ-receptor with subsequent Syk kinase and PI3Kinase activation mediates the IL-1Ra induction by chitin.

Chitin synergizes with Syk-independent-PRR pathways leading to IL-1β production that is dependent on immunoglobulins, Syk and PI3Kinase

Since the immune system is probably never confronted in nature with the highly purified chitin alone, but rather in the setting of different PRR ligands of the *A. fumigatus* conidia and hyphal cell wall, we stimulated PBMCs with chitin in the absence and presence of serum in combination with NOD2, TLR2, TLR4 and dectin-1 ligands. In the absence of serum no cytokines were induced. All non-Syk dependent PRR pathways stimulated with ligands, such as MDP (NOD2 pathway), Pam3Cys (TLR2 pathway) and LPS (TLR4 pathway) synergized with chitin to induce an higher IL-1β and TNFα production, while stimulation with β-glucan (dectin-1/Syk pathway) combined with chitin did not induce IL-1β (Fig 7A), TNFα (Fig 7B) or IL-6 (Supplemental Fig 1). In contrast, chitin did not have

synergistic effects on the LPS-induced IL-1Ra production; instead LPS combined with chitin induced IL-1Ra additively (Supplemental Fig 3). Next, we wanted to elucidate whether similar pathways responsible for IL-1Ra induction by chitin were mediating the synergistic response of chitin with other PAMPs. Using heat-inactivated serum we observed a significant higher ratio of LPS versus LPS/chitin-synergistic induction of IL-1β, while this ratio was significantly lower in the presence of Ig-depleted serum. Further heat-inactivation of the Ig-depleted serum did not change the ratio (Fig 7C). In addition, blocking Syk and PI3Kinase in PBMCs reduced the synergistic effect of chitin on LPS stimulation significantly, and the combination of both blockers completely inhibited the effect (Fig 7D). Collectively, these data suggest synergism induced by chitin is also immunoglobulin/FcγR dependent, and provide evidence for crosstalk between chitin-induced FcγR signalling and other PRR pathways, with the exception of dectin-1.

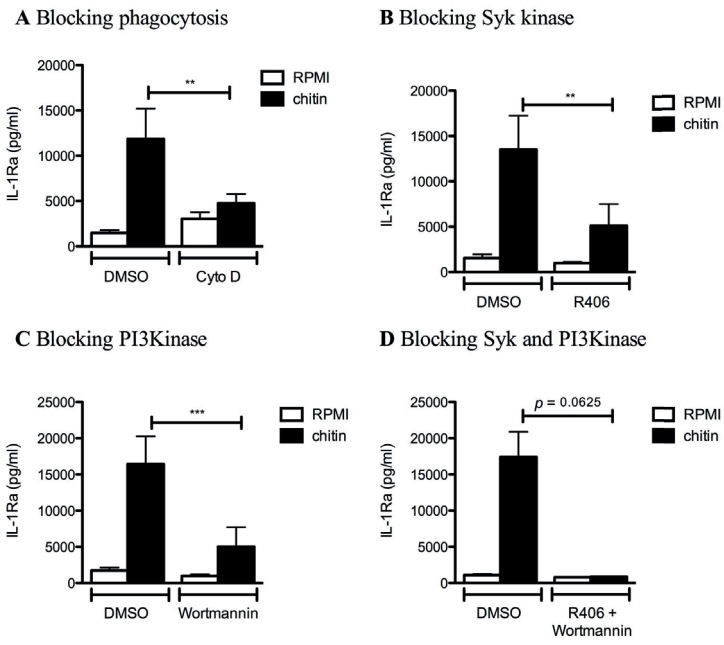

Figure 6: *Chitin-induced IL-1Ra is dependent on actin polymerisation and signals via Syk and PI3Kinase*

(A-D) PBMCs of healthy volunteers were stimulated with RPMI or chitin in the presence of (A) cytochalasin D actin depolymerisation agent (n = 13), (B) the Syk kinase inhitibor R406 (n = 10), (C) Wortmannin (n = 11) PI3K inhibitor or (D) the combination of R406 and Wortmannin (n=5), and IL-1Ra was measured in the cell culture supernatant by ELISA. Statistical analysis was performed with the Wilcoxon Signed Rank test (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

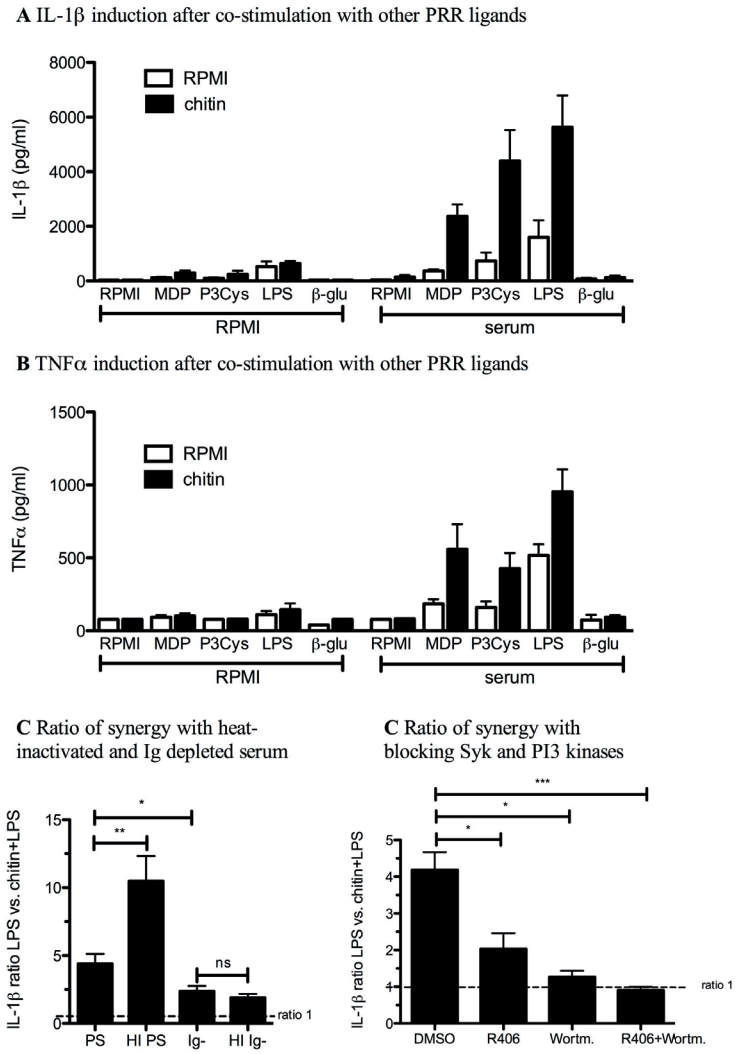

Figure 7: *Chitin synergizes with not-Syk-activating PRR ligands leading to high IL-1β production dependent on immunoglobulins, Syk and PI3Kinase*

PBMCs of healthy volunteers were stimulated with (A+B) chitin, mdp, Pam3Cys, LPS, β-glucan and with the combination of PRR ligands with chitin in the absence or presence of human poolserum (n = 5-18), or (C+D) with LPS and LPS together with chitin, either (C) in the presence of heat-inactived (HI) poolserum, Ig depleted serum (Ig-) or heat-inactivated Ig depleted serum (HI Ig-) (n=9), or (D) after blocking Syk kinase or PI3Kinase or the combination of both (n = 5-7). (C+D) The ratio between LPS-induced IL-1β and chitin + LPS-induced IL-1β was calculated. (A-D) IL-1β or TNFα (B) were measured in the cell culture supernatant by ELISA. Statistical analysis was performed with the Wilcoxon Signed Rank test (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

DISCUSSION

No pattern recognition receptor or signalling pathway activated by chitin has been identified in human immune cells to date, and immunostimulatory capacities range from absence of effects, to anti-inflammatory, and even pro-inflammatory properties (34). In the present study, we describe a mechanism by which chitin can induce immune responses in human immune cells and identify that chitin has both anti-inflammatory properties by inducing IL-1Ra and pro-inflammatory effects by inducing IL-1β/TNFα in synergy with Syk-independent PRR pathways. Interestingly, none of the previously proposed chitin receptors such as TLR2, MR, NOD2 or dectin-1 were involved in the induction of IL-1Ra in human PBMCs, but phagocytosis, recognition via the FcγRII with subsequent Syk- and PI3K-signalling of IgG opsonized chitin was identified as the signalling pathway. Strikingly, the effect was only observed with chitin particles in the presence of immunoglobulins. Therefore, we propose that chitin has a dual immunological function by dampening and controlling immune responses together with immunoglobulins via the induction of IL-1Ra on one hand, and orchestrating pro-inflammatory responses when chitin is presented in combination with PAMPs the other hand.

Purified *Aspergillus* chitin does not induce any pro-inflammatory cytokines in PBMCs both in the absence or presence of human serum. This is in line with former studies of Mora-Montes *et al.* and Bueter *et al.* (20, 34), which described chitin as an immunologically inert particle, not inducing TNFα, IL-1β, IL-6 or IL-10 production (20) or inflammasome activation (35). This was observed in several cell types, such as murine bone-marrow derived macrophages, M1 and M2 macrophages, as well as mouse peritoneal cells, mouse bone-marrow derived DCs and human PBMCs (35). Recently, it has been reported that chitin derived from different fungal species or crab shells induced IL-10 and TNFα in human PBMCs in a dose dependent manner (11). Several differences could account for this conflicting observation. The chitin particles in our study were mostly smaller than 1 μm, while the previously described IL-10 inducing chitin was between 1 and 10 μm (11). In addition, the use of *Aspergillus* to isolate chitin, and the different chitin purification method could account for the differences observed in cytokine responses. The presence of a contaminant even at low dose in the chitin sample can have a significant effect on the response to chitin since we have shown that polysaccharides can have an opposite effect than chitin on the immune response when tested in combination with chitin.

While former studies focused on IL-10 as a potent anti-inflammatory cytokine, less attention has been paid to another potent endogenous anti-inflammatory cytokine, namely IL-1Ra. In this study stimulation of PBMCs with chitin in the presence of human serum resulted in IL-1Ra transcription and production. The fine-tuned balance of pro- versus

anti-inflammatory cytokines of the IL-1 family is important during systemic inflammation and several reports point to a crucial role for a balanced IL-1 response to prevent *Aspergillus*-induced pathology (36-38).

Since the MR has previously been reported to be involved in chitin-induced immune responses in mice (11), we investigated mannose-binding lectin, which shares homology with the MR, as well as blocked the MR on PBMCs. However, MBL and MR were not involved in IL-1Ra induction by chitin. Although CR3 plays a crucial role in mediating adaptive T cell responses against *Aspergillus* conidia (17, 39), this receptor was also not involved in chitin-induced IL-1Ra. Moreover, stimulation of PBMCs of a kindlin-3 deficient patient (defective CR3 signalling (33)) with chitin revealed an intact IL-1Ra pathway. These data suggest that CR3 and MBL are not involved in chitin-induced IL-1Ra production. However, the fact that IL-1Ra induction by chitin was partly dependent on heat-inactivation suggests a role for complement system in the induction of IL-1Ra by chitin. Since Agarwal *et. al*. had shown in an earlier study that chitin is a poor inducer of the alternative complement pathway (40), the role of complement activation seen in this study could be caused by activation of the classical pathway. Elucidating this should be part of future studies.

In contrast to complement, immunoglobulins were essential for mediating the induction of IL-1Ra induced by chitin. Chitin showed direct interaction with immunoglobulins and the replenishment of IVIGs in Ig-depleted serum restored IL-1Ra induction. In addition, IL-1Ra was significantly lower when serum was depleted from chitin-binding immunoglobulins, suggesting that anti-chitin-specific IgGs mediates the IL-1Ra induction. Fcγ receptors can be activated by binding to immunoglobulin opsonized particles and subsequent internalisation, and receptor clustering (41). FcγRII has low affinity for monomeric IgG and only recognizes IgG complexes (42, 43), which might be similar to the ones artificially created by the binding of many IgG to long chitin molecules. Blocking FcγRII resulted in a significant decrease of the induction of IL-1Ra by chitin, suggesting that chitin opsonized with IgGs triggers FcγII receptor signalling, subsequently leading to IL-1Ra. Moreover, we identified that IL-1Ra induction of opsonized chitin was dependent on phagocytosis, Syk and PI3K activation.

Other polysaccharides present in the cell wall of *Aspergillus*, such as galactosaminogalactan and β-glucan can also induce IL-1Ra (36, 44). However, both polysaccharides do not require human serum for the induction of IL-1Ra. Interestingly the induction of IL-1Ra by β-glucan was shown to be independent of dectin-1, but dependent on PI3K (45). Therefore, it might be that the PI3K-Akt pathway is the final common pathway essential for IL-1Ra induction by polysaccharides, but that this pathway can be induced via different mechanisms depending on the ligand. Furthermore, it has been shown

that insoluble fibrillar polysaccharides can induce potent inflammatory responses by engaging multimerization of pattern recognition receptors (PRRs) and formation of supramolecular PRR complexes, which is known as the fibril hypothesis (46). We observed striking effects when cells were stimulated with opsonized chitin in the presence of other PRR ligands. Ligands for TLR2, TLR4 and NOD2 mediated potent synergistic effects on pro-inflammatory cytokines such as IL-1β. Interestingly, the combination of chitin with β-glucan, the ligand for dectin-1, did not induce IL-1β production, not even in the presence of human serum. Recently, crosstalk between Fcγ-receptors with other TLRs has been described (42). Vogelpoel *et al.* observed synergism with TLR2 and TLR4 ligands, but not with dectin-1 ligands, which is in line with the data from our study. Whether this is because Fcγ-receptors and dectin-1 both signal via Syk-PI3K-Akt needs to be further elucidated (47, 48). Since the synergism was dependent on IgGs and PI3K signalling, it is tempting to speculate that independent of a specific receptor, opsonized polysaccharides can induce potent synergistic pro-inflammatory responses via Fcγ-receptor activation when other pro-inflammatory ligands are present.

Studying the role of chitin-specific antibodies during aspergillosis might lead to new insights that could be relevant for the clinical setting. Indeed, an increase in anti-chitin antibodies have been shown in immunocompetent patients with chronic pulmonary aspergillosis and allergic aspergillosis (Raj and Latgé, unpublished). The role of the level of antibodies on the immune response towards chitin should be investigated in the future.

Another important question is how the opsonisation of chitin with specific antibodies could influence disease severity. Only little data exist, however Kin *et. al.* have shown that antibodies against bacterial polysaccharides can bind to chitin and dampen the overall immune response to chitin (49). In addition, since we provide evidence that chitin induces a different immune response when tested alone or with other PAMPs and since we know that the different polysaccharides of *A.fumigatus* will activate differently inflammatory responses, it is now of interest to test the effect of combinations of the cell wall polysaccharides to see if these combination are neutral, antagonist or synergistic.

In conclusion, we identified a novel mechanism through which chitin has strong anti-inflammatory effects and in the setting of other PAMPs can boost pro-inflammatory responses (Fig 8). These responses were critically dependent on immunoglobulins, Fcγ receptor signalling and phagocytosis. These data might explain, why the responses reported by chitin are so diverse. Moreover, since immunoglobulins might also influence other polysaccharide-induced immune responses, future studies need to focus on immunoglobulins and FcγR signalling when investigating fungal or fungal-cell wall component-induced immune responses.

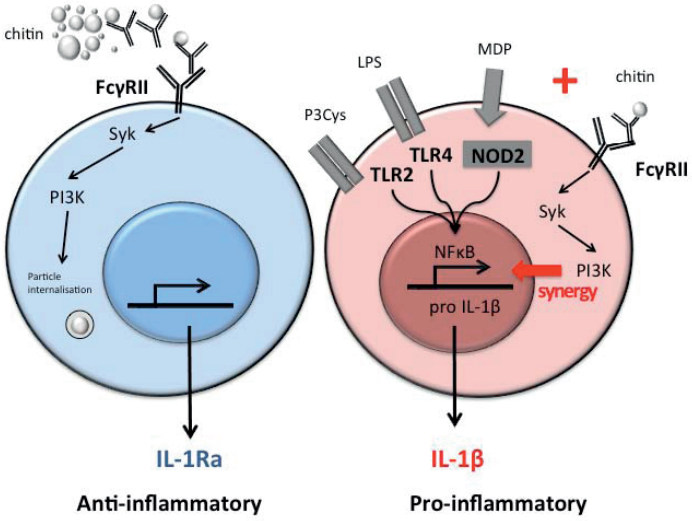

Figure 8: *Model of chitin-induced anti-inflammatory IL-1Ra and pro-inflammatory IL-1β responses*

IgG-opsonized chitin is recognized by the Fcγ-receptor and uptake induced via the Syk/PI3K-pathway, which results in isolated induction of IL-1Ra. In the presence of other Syk-independent PRR ligands, like P3Cys, LPS and MDP IgG-opsonized chitin induces IL-1β in a synergistic manner.

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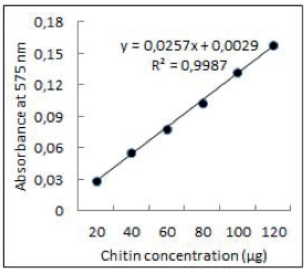
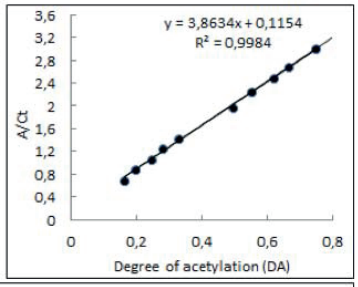
Chitin Conc, (µg)	Absorbance	Primary-NH	Primary-NH, %
20	0,029	1,0155642	5,077821012
40	0,056	2,06614786	5,16536965
60	0,078	2,92217899	4,870298314
80	0,103	3,89494163	4,868677043
100	0,132	5,0233463	5,023346304
120	0,158	6,03501946	5,029182879
Degree of deacetylation			5,005782534
Primary-NH calculated using the formula, x = (y-C)/m			
x = (absorbance - 0,0029)/0,0257			
Degree of acetylation = 100-DDA = 100-5,01= 94,99%			



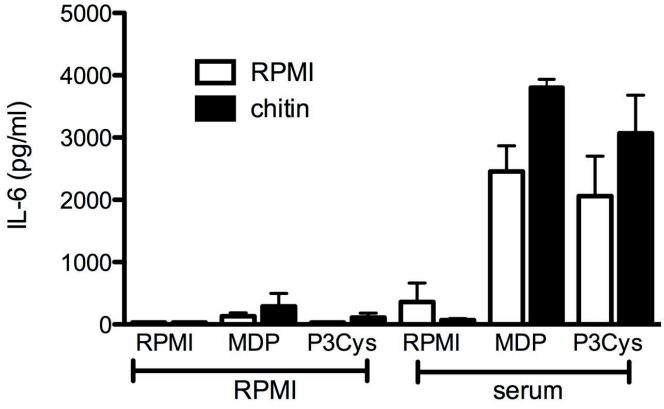
GlcNAc (mM)	GlcN (mM)	Ct	DA	A	A/Ct
0,27	0,09	0,36	0,75	1,09	3,00
0,27	0,14	0,41	0,66	1,10	2,68
0,23	0,14	0,37	0,62	0,91	2,49
0,23	0,19	0,41	0,55	0,92	2,24
0,18	0,19	0,37	0,49	0,73	1,99
0,18	0,37	0,55	0,33	0,79	1,43
0,09	0,23	0,32	0,28	0,40	1,25
0,09	0,28	0,37	0,25	0,39	1,06
0,05	0,19	0,23	0,20	0,20	0,88
0,05	0,23	0,28	0,16	0,19	0,70



Ct, total molar concentration [N-acetylglucosamine (GlcNAc)+glucosamine (GlcN)], DA, degree of acetylation, A = absorbance at 201 nm; DA = (161,1AV - 0,1154m)/(3,8634m-42,1AV), where m = chitin concentration and V = volume; Chitin samples were used at a concentration of 1 mg/ml; DA of two chitin tested were 93,4 and 89,3% (their average absorbances were 0,117 and 0,112, respectively)

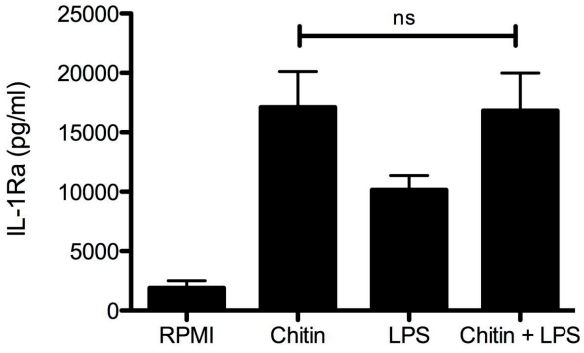
Supplemental Figure 1: *Measurement of the degree of acetylation of chitin*

With IR, UV spectrometric and dye binding measurements different batches of chitin showed degree of acetylation ranging between 89-94%. An average value of 91% for four different batches of chitin is presented.



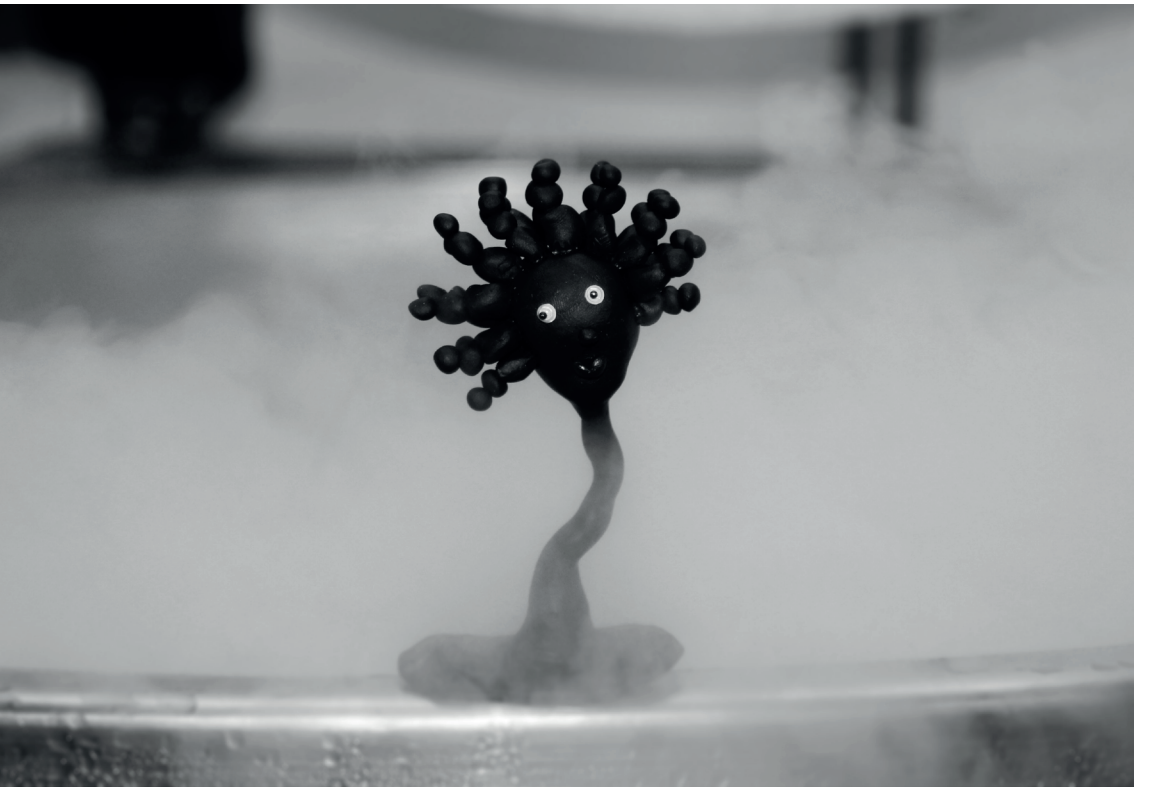
Supplemental Figure 2: *IL-6 induction after co-stimulation with other PRR ligands*

PBMCs of healthy volunteers were stimulated with chitin, mdp, Pam3Cys, and with the combination of PRR ligands with chitin in the absence or presence of human poolserum (n = 5-18).



Supplemental Figure 3: *No synergy of chitin and LPS for IL-1Ra*

PBMCs of healthy volunteers were stimulated with chitin, LPS and with the combination of chitin and LPS in the presence of human poolserum (n = 6). IL-1Ra was measured in the cell culture supernatant by ELISA. Statistical analysis was performed with the Wilcoxon Signed Rank test.



CHAPTER 4

Aspergillus fumigatus-induced IL-22 is not restricted to a specific Th cell subset and is dependent on complement receptor 3.

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ABSTRACT

T-helper cell responses induced by *Aspergillus fumigatus* have been extensively investigated in mouse models. However, the requirements for differentiation and the characteristics of *A. fumigatus*-induced human T-helper (Th) cell subsets remain poorly defined. We demonstrate that *A. fumigatus* induces Th1 and Th17 subsets in human PBMCs. Moreover, we show that the cytokine IL-22 is not restricted to a specific T-helper subset, in contrast to IL-17A. The pattern recognition and cytokine pathways that skew these *Aspergillus*-induced T-helper responses are TLR4- and IL-1-, IL-23-, and TNFα- dependent. These pathways are of specific importance for production of the cytokines IL-17A and IL-22. Additionally, our data reveals that the dectin-1/Syk pathway is redundant, and TLR2 has an inhibitory effect on *Aspergillus*-induced IL-17A and IL-22 production. Notably, blocking complement receptor 3 (CR3) significantly reduced *Aspergillus*-induced Th1 and Th17 responses, and this was independent on the activation of the complement system. CR3 is a known receptor for β-1,3-glucan, however blocking CR3 had significant effects on T-helper responses induced by heat-killed *Aspergillus* conidia, which have minimal β-glucan expression on their cell surface. Collectively, these data characterize the human T-helper subsets induced by *Aspergillus*, demonstrate that the capability to produce IL-22 is not restricted to a specific T cell subset, and provide evidence that CR3 might play a significant role in the adaptive host defence against *Aspergillus*, although the ligand and its action remains to be elucidated.

INTRODUCTION

The primary line of defence against *Aspergillus fumigatus* is mediated by neutrophils and other cells of the innate immune system (1). In addition to the innate immune response, adaptive T-helper (Th) responses also play a crucial role during invasive aspergillosis (IA). The Th1 response is associated with a protection in IA (2). However, conflicting data are reported regarding the role of Th17 responses in the host defence against *A. fumigatus.* A protective role for IL-17 is described by Werner *et al.* who showed that dectin-1$^{-/-}$ mice have decreased IL-17 production, and subsequently reduced *A. fumigatus* clearance (3, 4). In contrast, other studies show that IL-17 promotes inflammation and reduces resistance to the fungal infection (5, 6). Although these studies have investigated the role of IL-17 in mice, our group has previously reported that the human host response against *A. fumigatus* is mainly driven by the Th1 response, rather than the Th17 response (7). In addition, human mononuclear cells have been shown to express a primarily Th1 biased cytokine profile in response to stimulation with *Aspergillus* (8), and when Th2 responses are being suppressed enhanced protective Th1 response develop in mice (9). Furthermore, mice are resistant to *A. fumigatus* when proper IL-12 dependent Th1 responses are induced (9, 10).

Even though various reports have focused on Th1 and Th17 responses against *A. fumigatus,* a role for the cytokine IL-22, which is a characteristic cytokine of the Th17 response, has not been addressed in human host responses. Recently, a protective role for IL-22 was demonstrated in the early host defence against *A. fumigatus* in a murine model of invasive pulmonary aspergillosis (11). Furthermore, the induction of IL-22 contributes to lung pathology in a murine model of allergic bronchopulmonary aspergillosis (ABPA) (12). In the host defence IL-22 is primarily responsible for the induction of anti-microbial peptides (13) and is mainly produced by CD4^{+} T-cells, Natural Killer (NK) cells and NKT-cells (13-19). Within the CD4^{+} population, IL-22 is mainly produced by cells of the Th17 lineage (13), but also T-cells that are specialized in the production of IL-22 and TNFα; named Th22 cells (19).

Still, little is known about which cells mainly produce IL-17 (IL-17A), IFNγ and/or IL-22, and which recognition pathways and cytokines play a role in the induction of these cytokines in response to *A. fumigatus* in humans. In the present study we investigated the *A. fumigatus*-induced characteristic T-helper cytokines IL-17, IL-22, and IFNγ in PBMCs to elucidate which human cells primarily produce these cytokines, and which pattern recognition receptors and cytokines are involved in the induction of these cytokines in response to *A. fumigatus.*

METHODS

Healthy volunteers and patients

Blood samples from healthy controls and patients were obtained after written informed consent. Three patients with homozygous Y238X mutations in exon 6 of *CLEC7A* gene (the gene encoding dectin-1) provided blood samples. In these patients diminished dectin-1 expression and failure to induce a cytokine response to β-glucan was demonstrated previously (20).

Aspergillus

A clinical isolate of *Aspergillus fumigatus* V05-27, which was previously characterized (21), was used for all stimulations. Conidia and hyphae were prepared and heat-inactivated (HI) as described previously (22). A concentration of $1x10^7$/mL was used in the experiments.

β-1,3-glucan immunofluorescence of heat inactivated Aspergillus

To determine β-1,3-glucan expression after heat inactivation of *Aspergillus*. The HI conidia and hyphae were incubated for 30 minutes with mouse anti-β-1,3-glucan (Biosupplies, Bundoora, Australia). Subsequently the *Aspergillus* was washed and antibodies directed to β-glucan that were bound to *Aspergillus* were secondarily stained by goat anti-mouse alexa^{488} (Invivogen) according to the protocol supplied by the manufacturer. Immunofluorescence was observed at 400x magnification using a Zeiss Axio imager M1 fluorescence microscope, equipped with MRm camera (Carl Zeiss, Sliedrecht, The Netherlands).

PBMC isolation

Venous blood was drawn into 10 mL EDTA tubes, and PBMCs were isolated as described previously (21). In brief, blood was diluted in phosphate buffered saline (PBS) (1:1) and fractions were separated by Ficoll (Ficoll-Paque Plus, GE healthcare, Zeist, The Netherlands) density gradient centrifugation. Cells were washed twice with PBS and resuspended in RPMI-1640 culture medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 μg/mL gentamicin, 10mM L-glutamine and 10mM pyruvate (Gibco). The cells were counted using a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the cell concentration was adjusted to $5x10^6$/mL.

CD4/CD56 cell depletion

To deplete the CD56 or CD4 cells from isolated PBMCs, cells were labelled using magnetic beads coated with anti-CD56 or anti-CD4 (MACS Miltenyi, Bergisch Gladbach, Germany). Subsequently, the cells were depleted over a depletion column according to the protocol supplied by the manufacturer. As control for the isolation procedure PBMCs were also runned over the columns without labelling with magnetic beads.

PBMC stimulation

PBMCs were plated in 96-well round bottom plates (Corning, NY, USA) at a concentration of $2.5x10^6$/mL in a volume of 200 mL. They were either not stimulated or stimulated with $1x10^7$/mL HI conidia or hyphae for 24 hours or 7 days at 37˚C and 5% CO_2. All stimulations were performed in medium containing 10% human serum, which was obtained from a serum pool of healthy volunteers.

Pattern recognition receptors were inhibited in PBMCs by pre-incubation for 1 hour with specific inhibitors. LPS derived from *Bartonella quitana* was used to block TLR4 at a final concentration of 20 ng/mL (23). *B. quitana* LPS was extracted and purified as described previously (24). Mouse anti-humanTLR-2 (eBioscience, Halle-Zoersel, Belgium) and control mouse IgG1 (eBioscience), anti-human integrin β2 (αCR3) and control goat IgG (R&D systems Minneapolis, MN, USA) were used in a final concentration of 10 µg/mL. Laminarin was kindly provided by Professor David Williams of Tennessee University and was used in a final concentration of 50 ng/mL to inhibit dectin-1. Syk kinase inhibitor was purchased from Calbiochem (Merck, Darmstadt, Germany) and was used in a concentration of 50 nM. In order to check the blockade of the PRRs, PBMCs were stimulated with the TLR4 ligand LPS (10 ng/mL) from *Escherichia coli* serotype O55:B5 (Sigma Chemical Co, St Louis, MO, USA); or with the TLR2 ligand Pam3Cys (1 µg/mL) (EMC microcollections, Tübingen, Germany). Inhibition of dectin-1 and Syk was validated by stimulation with HI *C. albicans* ($1x10^6$/mL). After 24 hours of stimulation at 37˚C and 5% CO_2 IL-1β was measured by ELISA. All blockades resulted in a significant reduction of cytokine production (Supplementary figure 1A, B).

The cytokine pathways of IL-1, IL-23 and TNFα were investigated using supplementation of the cultures with recombinant human (rh) IL-23 (50 ng/mL) and rhTNFα (10 and 100 ng/mL) (R&D Systems). IL-1 receptor signalling was blocked by its natural receptor antagonist (Ra) IL-1Ra (10 µg/mL) (Amgen, Inc., Thousand Oaks, CA, USA), and IL-23 was blocked with mouse anti-human IL-23p19 (10 µg/mL) (R&D systems). sTNFRII (Enbrel) and human anti-human TNFα (Humira) were kindly provided by Dr. Renoud Marijnissen and Dr. Marije Koenders of the department of Rheumatology Radboud University Nijmegen

Medical Centre the Netherlands, and were used to block TNFα in a final concentration of 100 µg/mL.

Cytokine measurements

IL-17A, IL-22, IFNγ, IL-1β and IL-23 were measured using commercially available ELISAs (R&D systems or eBioscience) according to the protocol supplied by the manufacturers.

Intracellular IL-17, IL-22, and IFNγ flowcytometry

Following 7 days stimulation, PBMCs were stimulated 4-6 hours with PMA (50 ng/mL) (Sigma-Aldrich), ionomycin (1 µg/mL) (Sigma-Aldrich) and Golgiplug (BD Biosciences, Breda, the Netherlands) according to the protocol supplied by the manufacturer. Cells were stained extracellular using PeCy7-conjugated anti-CD4 (BD Biosciences), PeCy7-conjugated anti-CD8 (Biolegend, San Diego, CA, USA) or PeCy7-conjugated anti-CD56 (Beckman Coulter) antibody. Subsequently the cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the protocol supplied by the manufacturer. Following permeabilization the cells were stained intracellular with alexa^{647} conjugated anti-IL-17 (BD Biosciences), PE conjugated anti-IL-22 (R&D systems) and FITC conjugated anti-IFNγ (eBioscience) according to the protocol supplied by the manufacturers. The cells were measured on a FC500 flowcytometer (Beckman Coulter) and the data were analysed using CXP analysis software v2.2 (Beckman Coulter).

Statistical Analysis

Differences in IL-17, IL-22, and IFNγ production and the percentage of CD4$^+$ cells between the medium and *Aspergillus* stimulated samples were analysed with the Mann-Whitney-U test. Data of stimulations with and without inhibitors of PRRs, cytokines, or cytokine inhibitors were subjected to statistical analysis with the Wilcoxon signed rank test. A p-value of <0.05 was considered statistically significant, with $p<0.05$ = *, $p<0.01$ = **, and $p<0.001$ = ***. All experiments were performed at least twice and data represent cumulative results of all experiments performed and are presented as mean ± standard error of the mean (SEM) unless indicated otherwise. Data were analysed using GraphPad Prism v 5.0. The proportional Venn diagram was drawn using the eulerAPE application v2.0.3. (25, 26)

RESULTS

The proinflammatory adaptive cytokine response to *A. fumigatus*

We investigated the capacity of *A. fumigatus* conidia and hyphae to induce the cytokines IL-17A, IL-22 and IFNγ. Stimulation with conidia and hyphae induced a significant production of the Th1 cytokine IFNγ in human PBMCs, whereas IL-17A was induced in low amounts. Both conidia and hyphae also induced IL-22 in human PBMCs (Figure 1A).

To determine which cell populations expand after stimulation with *Aspergillus,* we performed flowcytometry analysis. We initially focused on $CD4^+$ cells, since T-helper cells are generally considered to be the main producers of IL-17A, IL-22 and IFNγ. PBMCs that were not stimulated with conidia showed relatively small populations of $IL\text{-}17A^+$ and $IL\text{-}22^+$ CD4 cells (1.2% SD 0.7 and 1.7% SD 0.9 respectively), whereas the $IFN\gamma^+$ CD4 cell population was 10.7% (SD 3.9). Stimulation with conidia induced a significant expansion of the $IL\text{-}17A^+$, $IL\text{-}22^+$ and $IFN\gamma^+$ CD4 T-cell population (Figure 1B). By gating on the *Aspergillus*-induced $IL\text{-}17A^+$, $IL\text{-}22^+$ and $IFN\gamma^+$ cells we observed that the majority of $IL\text{-}17A^+$ and $IL\text{-}22^+$ cells were $CD4^+$ cells. However, approximately half of the $IFN\gamma^+$ cells were negative for CD4 (Figure 1C). Subsequently, extracellular staining with the NK-cell marker CD56 and the cytotoxic T-cell marker CD8 revealed that a significant proportion of to the $IFN\gamma^+$ population was $CD56^+$ and $CD8^+$ (figure 1D). The remainder of the $IL\text{-}17A^+$ cells was positive for $CD8^+$ only. No $CD56^+$ $IL\text{-}17A^+$ cells were found (figure 1D). However, despite the fact that a fraction of the $IL\text{-}22^+$ cells was positive for CD8 or CD56, still approximately 9% of the $IL\text{-}22^+$ was negative for all the investigated surface markers (figure 1D). Interestingly, we found that the populations of $CD8^+$ and $CD56^+$ cells that were $IFN\gamma^+$ were already present in unstimulated PBMCs, and did not expand upon stimulation with *A. fumigatus* (figure 1E).

CD4 cells are the primary cellular source of *Aspergillus*-induced IL-17A, IL-22 and IFNγ

To demonstrate that $CD4^+$ cells are the major contributors to *Aspergillus*-induced IL-17A, IL-22 and IFNγ, we depleted $CD4^+$cells from PBMCs and compared this with normal PBMCs. Depletion of $CD4^+$ cells resulted in a complete loss of IL-17A production. Furthermore, IL-22 and IFNγ production were also significantly reduced, in most donors, to undetectable levels (figure 1F). Since several reports indicate that NK-cells can produce IL-22 (14, 16-18), we also investigated the contribution of $CD56^+$ cells. Depletion of $CD56^+$ cells had no significant effect on IL-17A, IL-22 and IFNγ production (Figure 1G).

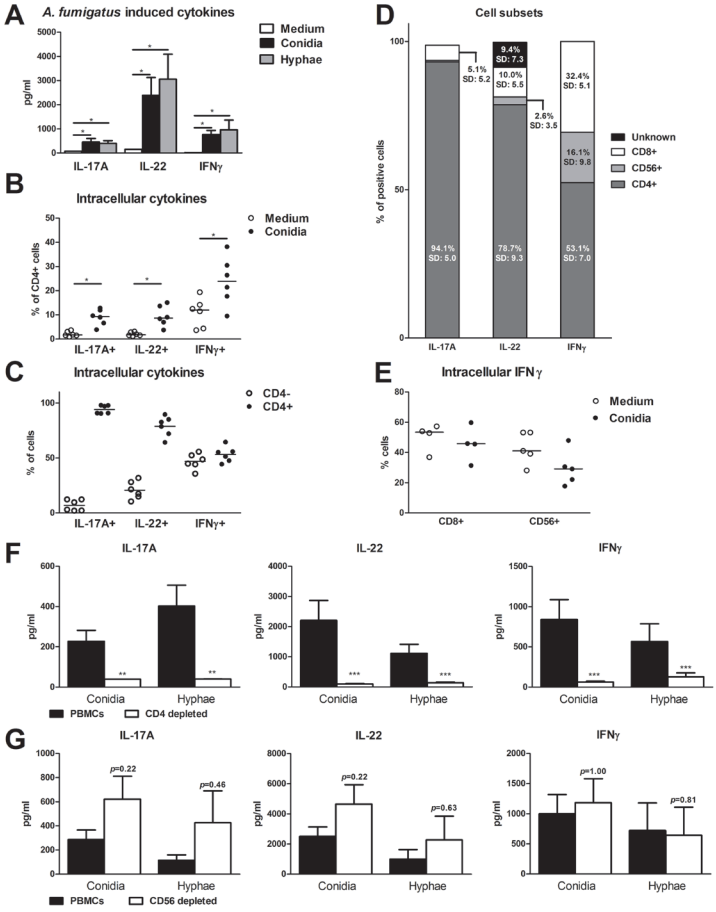

Figure 1 Induction of IL-17A, IL-22, and IFNγ by *A. fumigatus,* and the cellular source of these cytokines

(A) IL-17A, IL-22 and IFNγ concentrations in culture supernatants of PBMCs (2.5x10^6/mL) (n=6 donors) that were stimulated with 10^7/mL HI *A. fumigatus* conidia or hyphae in the presence of 10% human serum. (B) Intracellular IL-17A, IL-22 and IFNγ in CD4 T-cells in the PBMCs of the experiments shown in panel A. (C) The percentage of $CD4^-$ and $CD4^+$ cells within the IL-17, IL-22 and IFNγ positive populations in *Aspergillus*-stimulated PBMCs of the experiments shown in panel a. (D) Assessment of surface markers CD56 and CD8 to elucidate the contribution of different cell types to the population of $IL\text{-}17A^+$, $IL\text{-}22^+$ and $IFN\gamma^+$ cells. (E) The expansion of $CD8^+$ $IFN\gamma^+$ and $CD56^+$ $IFN\gamma^+$ was determined by comparing unstimulated PBMCs to *A. fumigatus* stimulated PBMCs (n=4 for CD8 and n=5 for CD56). (F, G) IL-17A, IL-22 and IFNγ concentrations in culture supernatants of PBMCs, (F) PBMCs depleted of $CD4^+$ cells (n=10 donors for IL-17, n=12 donors for IL-22 and n=11 for IFNγ), (G) PBMCs depleted of $CD56^+$ cells (n=6 donors) that were stimulated with 10^7/mL HI *A. fumigatus* conidia or hyphae in the presence of 10% human serum. The Wilcoxon signed rank test was used to determine whether the means were significantly different.

IL-22 is not restricted to a specific T-helper subset

Since CD4$^+$ cells were the major population that were IL-17A$^+$, IL-22$^+$ and IFNγ$^+$ we investigated the phenotypic diversity of these CD4$^+$ T-cells by focusing on the expression of single or multiple cytokines. Stimulation with HI conidia resulted in the induction of IL-17/IL-22 and IL-22/IFNγ double positive CD4 cells (Figure 2A). Whereas we found a relatively small percentage of IL-17/IFNγ double positive CD4$^+$ T-cells. Strikingly, the cytokine IL-22 was not specifically expressed in a certain subset, but could be found in both the IL-17 and IFNγ positive T-cell populations. Moreover, A*spergillus* induces IL-17/IL-22/IFNγ triple positive CD4$^+$ T-cells (Figure 2A). To determine whether the IL-22$^+$ cells in our experiments match the phenotype of previously-described Th22 cells we

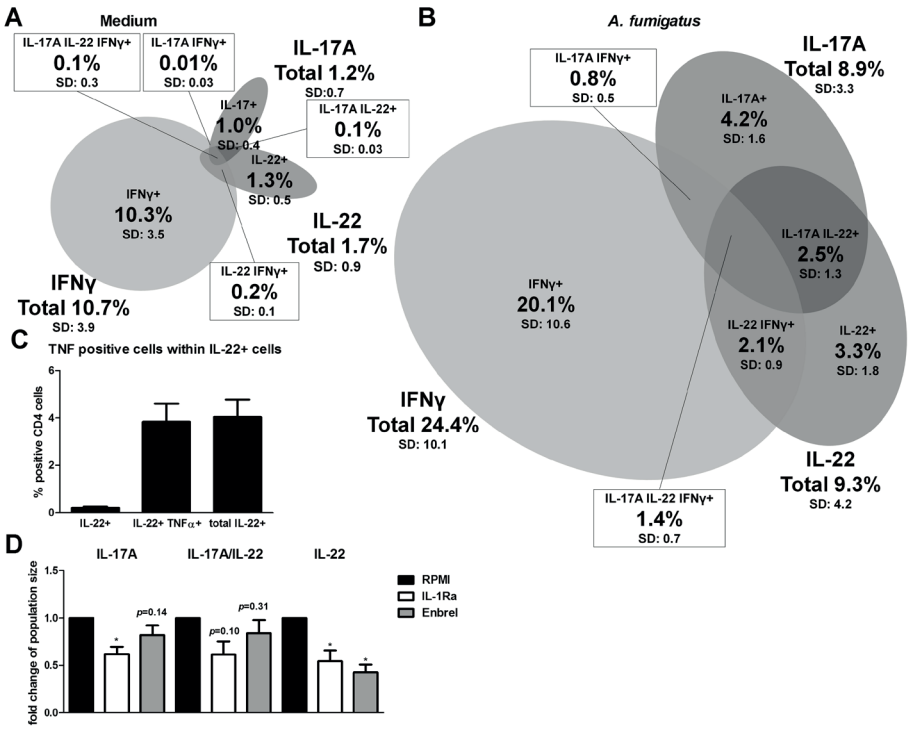

Figure 2 Phenotypic diversity of IL-17A, IL-22 and IFNγ positive CD4 T-cells

Proportional Venn diagrams of IL-17A, IL-22, and IFNγ positive CD4 cell populations of (A) medium stimulated PBMCs and (B) *Aspergillus* conidia-stimulated PBMCs. Overlap between the ellipses represent double and triple positive CD4 cells. (C) Expression of TNFα within the IL-22$^+$ cell population of *Aspergillus*-stimulated PBMCs. (D) IL-17$^+$, IL-17/IL-22$^+$ and IL-22$^+$ CD4 cell populations of *Aspergillus* conidia- stimulated PBMCs (n=6 donors) in the presence or absence of IL-1Ra or sTNFRII (Enbrel) (Data represented as fold change from stimulation in absence of inhibitor).

assessed whether these cells co-expressed TNFα, a characteristic of Th22 cells (15, 19, 27). Indeed, the majority of IL-22$^+$ cells co-expressed TNFα (Figure 2B). Additionally, we investigated how IL-1 and TNFα signalling can influence the IL-17 and IL-22 populations. Blocking IL-1 resulted in significantly decreased IL-17$^+$ and IL-22$^+$ populations and a trend towards decreased IL-17/IL-22$^+$ cells, whereas blocking TNFα with sTNFRII only significantly decreased the number of IL-22$^+$ cells (Figure 2C).

Differential roles for TLR2 and TLR4 in *Aspergillus*-induced T-cell responses

We investigated the role of the Toll-like receptors in the induction of the IL-17, IL-22, and IFNγ responses to *A. fumigatus,* as TLR2 and TLR4 have been associated with the recognition of *A. fumigatus* (28-32). PBMCs were pre-incubated with *B. quintana* LPS to block TLR4, and afterwards stimulated with conidia or hyphae. Pre-incubation with *B. quintana* LPS alone did not result in any cytokine induction in PBMCs (Supplementary Figure 1C). Blockade of TLR4 resulted in a significantly reduced IL-22 production in conidia-stimulated PBMCs and reduced IL-17 and IL-22 production in hyphae-stimulated PBMCs,

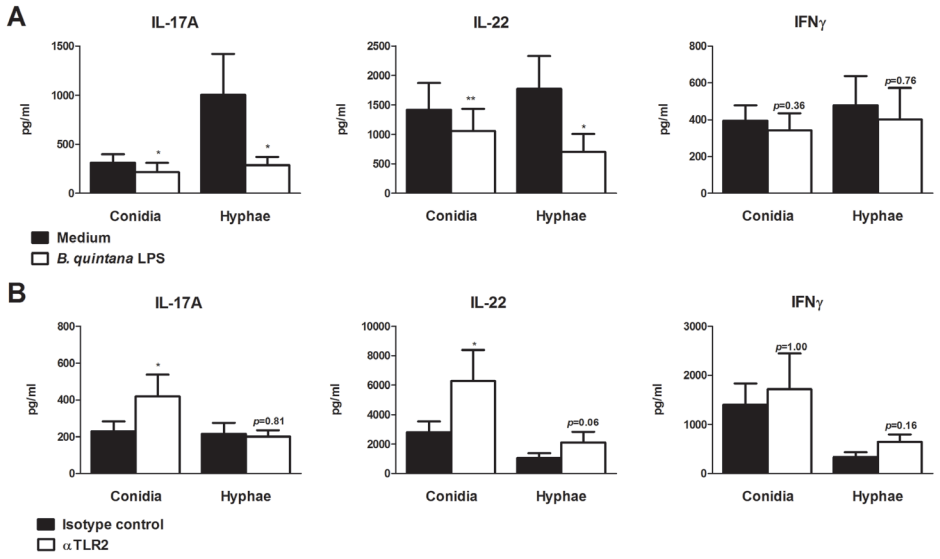

Figure 3 Differential roles for TLR4 and TLR2 in *Aspergillus* induced T-cell responses

(A) IL-17, IL-22 and IFNγ were measured in culture supernatants of PBMCs (2.5x10^6/mL) stimulated with 10^7/mL *A. fumigatus* conidia (n=7 for IL-17, n=8 for IL-22, and N=9 for IFNγ) or hyphae (n=7 for IL-17, n=7 for IL-22, and n=11for IFNγ) for 7 days, that were pre-incubated with *B. quintana* LPS to block TLR4 and were compared to pre-incubation with culture medium. (B) Similarly, TLR2 was blocked by 1 hour pre-incubation using mouse anti-human TLR2 monoclonal antibody which was compared to the isotype control (n=6).

whereas IFNγ production was not affected by TLR4 blockade (Figure 3A). Blocking TLR2 resulted in an upregulation of IL-17 and IL-22 production by conidia stimulated PBMCs and a trend towards upregulation of IL-22 in hyphae-stimulated PBMCs (Figure 3B).

Redundant role for dectin-1-Syk signalling in *Aspergillus*-induced T-cell responses

Dectin-1 has been associated with the defence against *Aspergillus* in numerous reports (3, 4, 33-38). Gessner and co-workers described that dectin-1 deficient mice lack the ability to induce IL-22 early in infection, which causes a decreased ability to induce antimicrobial peptides, resulting in an increased fungal burden and mortality (11). To see whether the dectin-1-Syk signalling pathway is involved in the induction of IL-22 production by human T-cells, PBMCs were stimulated with conidia or hyphae in the presence of laminarin, a dectin-1 inhibitor. Blocking dectin-1 with laminarin did not result in any significant effect on hyphae or conidia-induced IL-17A, IL-22, or IFNγ (Figure 4A). However, there was one donor with unusual high IL-17 production in response to hyphae, which we excluded from analysis. It should be noted that the IL-17 production in this donor was almost completely blocked with laminarin.

We investigated whether *Aspergillus* conidia and hyphae expressed β-1,3-glucan on the surface by immunofluorescence staining. We found that β-1,3-glucan was only minimally present on a few conidia, which is in line with reports demonstrating that resting conidia do not express β-glucan (34). In contrast, hyphae abundantly exposed beta-glucan (figure 4B). To confirm that dectin-1/Syk signalling does not play a significant role in the induction of IL-17A, IL-22 or IFNγ by *Aspergillus,* PBMCs of three patients with the homozygous mutation Y238X in dectin-1 (lacking dectin-1 expression on the membrane of the cell (20)) were stimulated with conidia and hyphae. PBMCs from these patients demonstrated either a similar or higher cytokine response to *Aspergillus* compared to PBMCs from a healthy control (Figure 4C).

Syk kinase is the downstream signalling kinase of dectin-1 and other C-type lectin receptors (CLRs) (34). Although, hyphae-induced IL-17 production tended to decrease upon blocking of dectin-1, inhibition of Syk kinase demonstrated no difference in IL-17A, IL-22, or IFNγ production by *Aspergillus* stimulated PBMCs (Figure 4D).

The role for complement receptor 3 (CR3) for *Aspergillus*-induced T-cell responses

Recently, CR3 was demonstrated to recognize β-glucan, which leads to downstream signalling and activation of granulocytes (39). Moreover, CR3 has also been associated with phagocytosis of pentraxin3 opsonised *Aspergillus* (40). We investigated whether

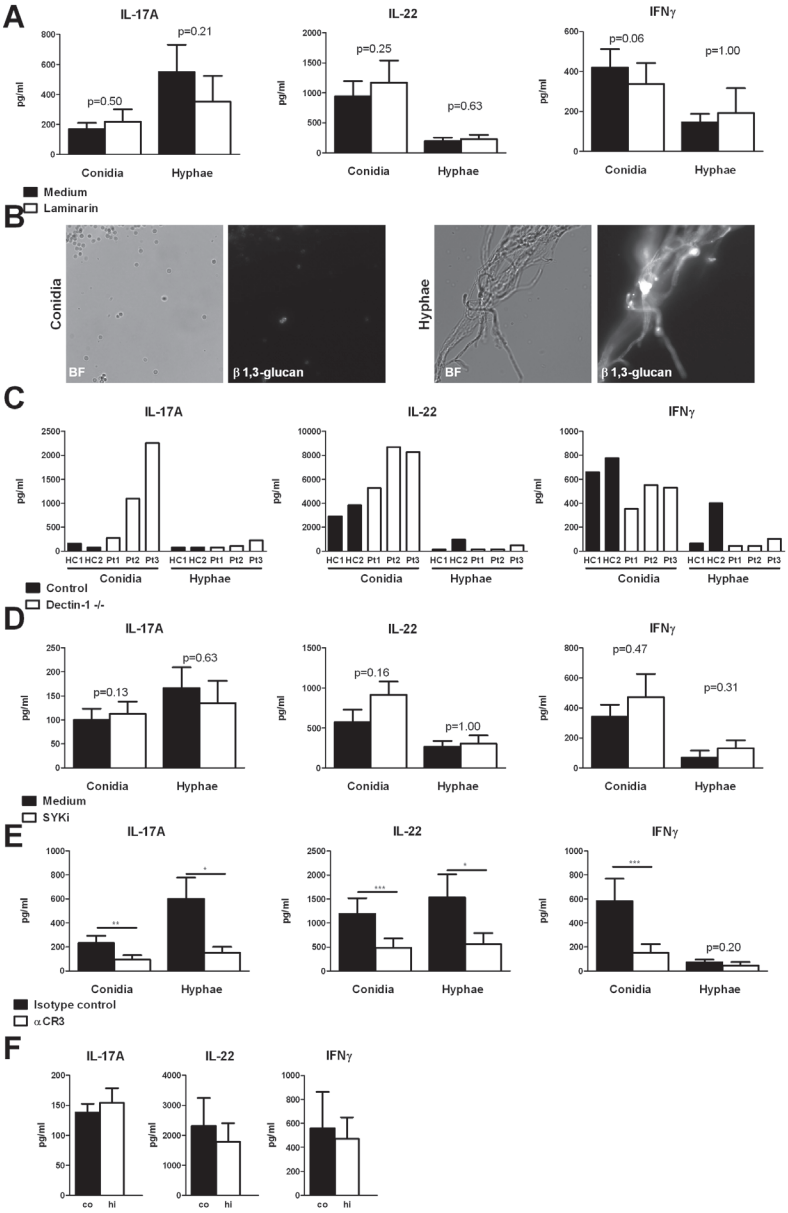

Figure 4 Redundant role of dectin-1 and Syk signalling in *Aspergillus*-induced IL-17A, IL-22, and IFNγ

(A) IL-17A, IL-22 and IFNγ in culture supernatants of PBMCs (2.5x10^6/mL) with 10^7/mL *A. fumigatus* conidia or hyphae for 7 days, that were pre-incubated with laminarin to block dectin-1, (n=8 for conidia and n=5 for hyphae). (B) Bright field (BF) and Immunofluorescence images after staining with mouse anti-β-1,3-glucan

conjugated with goat anti-mouse IgG alexa^{488}. HI *Aspergillus* conidia (left panels) and HI *Aspergillus* hyphae (right panels) at 400x magnification and an exposure time of 900ms for fluorescence pictures. (C) IL-17A, IL-22 and IFNγ in culture supernatants of PBMCs (2.5x10^6/mL) from three patients with a homozygous dectin-1 Y238X polymorphism and two healthy controls without the polymorphism were stimulated with *A. fumigatus* conidia or hyphae for 7 days and IL-17, IL-22, and IFNγ were measured in the culture supernatant. IL-17, IL-22 and IFNγ in culture supernatants of PBMCs (2.5x10^6/mL) stimulated with 10^7/mL *A. fumigatus* conidia or hyphae in presence or absence of (D) Syk kinase inhibitor (n=11 for conidia and n=6 for hyphae), or (E) goat anti-CR3 monoclonal antibody or an isotype control (conidia n=11 or hyphae n=6 for IL-17 and IL-22, n=8 for IFNγ). (F) IL-17, IL-22 and IFNγ in culture supernatants of PBMCs (2.5x10^6/mL) (n=6 donors) with 10^7/mL *A. fumigatus* conidia for 7 days in the presence of 10% human serum or 10% human serum that was heat inactivated for 30 minutes at 56°C. Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

CR3 was involved in the induction of IL-17A, IL-22, and IFNγ by *A. fumigatus*. Blockade of CR3 resulted in a significant reduction of IL-17A, IL-22 and IFNγ responses induced by conidia. In response to hyphal stimulation, only IL-17A and IL-22 were significantly inhibited by blocking of CR3 (Figure 4D). Since the primary role of CR3 is recognition of C3 opsonised structures, we investigated whether active serum complement was required for *Aspergillus*-induced IL-17A, IL-22 and IFNγ. When human serum was heat-inactivated prior to stimulation, no differences in IL-17A, IL-22 and IFNγ induction were observed (Figure 4E).

The role of IL-1 and IL-23 in *A. fumigatus*-induced IL-17 and IL-22

The predominant cytokine produced by human PBMCs in response to *Aspergillus* was IL-22, while the IL-17A production was relatively low (Figure 1A). To elucidate the reason for the low IL-17A release by *Aspergillus* stimulated PBMCs we focused on the cytokines that regulate the Th17 response. Both IL-1β and IL-23 have been associated with the induction and maintenance of the Th17 response in humans (41). Additionally, a critical role for IL-23 in the induction of IL-22 was observed in the early response against *A. fumigatus* (11). Interestingly, IL-23 induction by PBMCs stimulated with conidia and hyphae was below the detection limit of the ELISA, while IL-1β production was detectable (Figure 5A). To investigate whether the absence of IL-23 production is responsible for the low production of IL-17 in response to *Aspergillus*, IL-23 was blocked with an anti-human IL-23p19 antibody. Compared to stimulation with the isotype control, IL-17A production was reduced to undetectable levels although this was not statistically significant, while IL-22 production was not affected (Figure 5B). Addition of IL-23 significantly increased IL-17A production but did not increase IL-22 production (Figure 5C). Blocking IL-1 signalling with IL-1 receptor antagonist (IL-1Ra) reduced IL-17A and IL-22 induction significantly

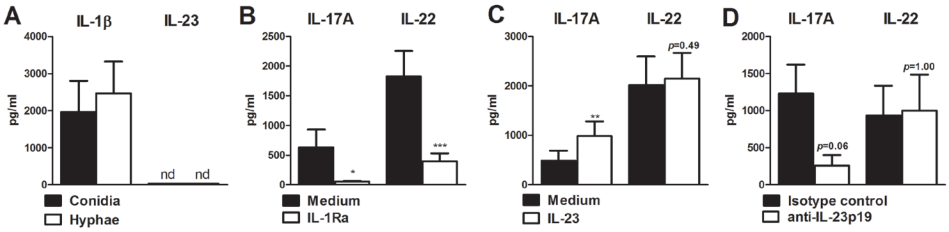

Figure 5 Role of IL-23 and IL-1 in *A. fumigatus* induced IL-17A and IL-22

IL-1β and IL-23 concentrations in PBMCs (2.5x10^6/mL) (n=7) that were stimulated with HI *A. fumigatus* conidia or hyphae 37°C and 5% CO_2 for 24 hours (A). IL-17 and IL-22 were measured in the culture supernatants of PBMCs (2.5x10^6/mL) stimulated with HI *A. fumigatus* conidia in the presence or absence of 10 µg/mL IL-1Ra (C) (n=7 for IL-17 n=12 for IL-22), IL-23 (D) (n=12), or anti-IL-23p19 (E) (n=5 for IL-17 n=6 for IL-22). Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

(Figure 5D). These results suggest that IL-17A production requires both IL-1β and IL-23, whereas IL-22 production requires only IL-1β.

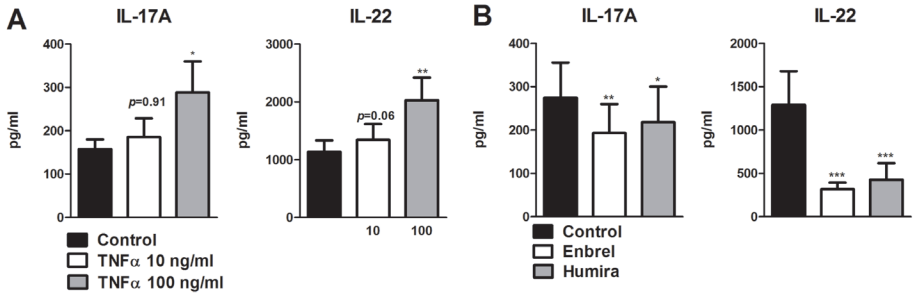

Figure 6 Role of TNFα in *A. fumigatus* induced IL-17A and IL-22

(A) IL-17A and IL-22 were measured in the culture supernatants of PBMCs (2.5x10^6/mL) (n=10 donors) stimulated for 7 days with 10^7/mL HI *A. fumigatus* conidia with or without recombinant human TNFα. (B) TNFα was blocked by pre-incubation of PBMCs (n=11) for 1 hour with sTNFRII (Enbrel) or human anti-TNFα (Humira). Subsequently the capacity to induce IL-17A and IL-22 was investigated by 7 days stimulation with HI *A. fumigatus* conidia. Differences of the means were analysed for significance using the Wilcoxon Signed Rank test.

Role of TNFα in *A. fumigatus*-induced IL-17 and IL-22

Although IL-1β and IL-23 play key roles in the induction of T-helper responses by *A. fumigatus*, it cannot be excluded that other cytokines also play a role in the induction of the cytokines IL-17 and IL-22. To investigate the role of TNFα in *A. fumigatus* induced IL-17 and IL-22 we stimulated PBMCs with HI conidia in the presence of 10 or 100 ng/mL TNFα. Both IL-17 and IL-22 production were dose dependently increased in the presence of TNFα when compared to stimulation with *Aspergillus* alone (Figure 6A). To further investigate the role of TNFα we stimulated PBMCs with HI conidia in the presence of sTNFRII (Enbrel) or human anti human TNFα (Humira), two drugs that are used to block TNFα signalling in IL-17 related diseases like rheumatoid arthritis (RA) (42). Both blockers significantly reduced the IL-17 and IL-22 response (Figure 6B).

DISCUSSION

In the present study we demonstrate that $CD4^+$ T cells are the main producers of IL-17, IL-22 and IFNγ in human PBMCs upon *Aspergillus* stimulation. Interestingly, CD4 T-helper cells that are capable of producing IL-22 after *Aspergillus* stimulation are not a distinct characteristic subset, but can have a Th1, Th17, or Th22 signature. Similar to IL-17, the production of IL-22 induced by *Aspergillus* is dependent on TLR pathways. While the TLR4 pathway contributing to the production of these cytokines, the TLR2 has an inhibitory effect on *Aspergillus*-induced IL-17 and IL-22 production. In contrast, the TLR2 and TLR4 pathway do not modulate IFNγ production by human PBMCs that are stimulated with conidia. Rather unanticipated, the blockade of the dectin-1/Syk pathway or absence of the dectin-1 receptor did not significantly affect IL-17, IL-22 or IFNγ production induced by *Aspergillus*. In the current study, CR3 is the only receptor that is important for induction of all three cytokines IL-17, IL-22 and IFNγ, and therefore the CR3 pathway could specifically play an important role in the host defence against *Aspergillus*. Furthermore, we provided evidence that IL-17 and IL-22 responses induced by *Aspergillus* are dependent on the IL–1 and TNF pathway.

Using flowcytometric analysis of PBMCs and depletion of CD4 or CD56 cell subsets, we determined that the cytokines IL-17, IL-22 and IFNγ are primarily produced by $CD4^+$ T-cells. Within the $CD4^+$ T-cell populations diverse intracellular cytokine expression was detected. We detected IL-17 single positive and IL-17/IL-22 double positive CD4 cells that match the classically described Th17 cells (13) and IFNγ positive CD4 cells that match the classical Th1 type. Half of the $IFN\gamma^+$ cells were $CD4^+$ cells, whereas the rest of the $IFN\gamma^+$ cells were negative for this T-helper-cell marker. We were able to demonstrate that a significant number of natural killer (NK) cells express IFNγ, which is in line with a

previous report that demonstrated that NK cells play an important role in the antifungal response to *A. fumigatus* by releasing IFNγ (43). However, depletion of $CD56^+$ cells did not alter IFNγ responses, suggesting that NK cells are not important for the production of IL-22 upon stimulation with *Aspergillus*. Furthermore, the $IFN\gamma^+$ $CD56^+$ cells did not expand upon stimulation with *Aspergillus.* These data suggest that, in the setting of PBMCs, NK cells do not contribute to the IFNγ response to *Aspergillus.* We found that after stimulation with *Aspergillus* IL-17/IFNγ double positive $CD4^+$ cells were present in low numbers. Therefore, our results demonstrate that the polarization of T-helper responses induced by *Aspergillus* in-vitro is different from that induced by the commensal fungus *Candida albicans* (44). It was demonstrated that *C. albicans* induces a T-cell polarization with high numbers of IL-17/IFNγ double positive T-helper cells. Here we demonstrate that *Aspergillus* stimulation leads to low numbers of this T cell subset. This is of interest, since these double positive cells have been linked to autoimmunity (44).

We also observed IL-22 single positive cells that might fit the description of Th22 cells (15), as these cells co-expressed TNFα. In addition, large numbers of IFNγ/IL-22 double positive CD4 cells and even IL-17/IL-22/IFNγ triple positive CD4 cells are present. This is to our knowledge the first study that demonstrates that *Aspergillus* can induce single $IL\text{-}22^+$ and IFNγ/IL-22 double positive cells. It remains to be elucidated whether these specific subsets contribute to pathology or protection. A study in mice revealed an important role for IL-22 in the early defence against *Aspergillus* (11), while another study demonstrates contribution to pathology in an allergic aspergillosis model (12). In the current study, we observed that the proinflammatory adaptive cytokine response to *A. fumigatus* is dominated by IL-22, and the primary cellular sources were $CD4^+$ cells. In contrast to the study performed by Gessner and co-workers, our model revealed that IL-22 production by *Aspergillus*-stimulated PBMCs was independent of IL-23. We found that the innate cytokines IL-1 and TNFα played a crucial role in the induction of IL-22, which is in agreement with earlier studies (45). Previously, it was demonstrated that the IL-22 response also dominated over the IL-17 response in patients with pulmonary tuberculosis (46). Moreover, IL-22 was shown to be important in the defence against multiple pulmonary pathogens including *Mycobacterium tuberculosis* (14, 46, 47), *Klebsiella pneumonia* (48), and influenza A virus (16). Since the host defence against all these pulmonary pathogens strongly relies on IL-22, we hypothesize that IL-22 plays a pivotal role in the human anti-*Aspergillus* pulmonary host defence.

Although TLR2 and TLR4 play an important role in innate immune responses against *A. fumigatus* (28-32), the role of these receptors in the induction of the adaptive immune responses such as the IL-22 response against *Aspergillus* remains to be established. Here we demonstrate that TLR4 plays a role in the induction of IL-17 and IL-22 in re-

sponse to *Aspergillus.* In contrast, blockade of TLR2 resulted in higher IL-17 and IL-22 production. Therefore, this might indicate that TLR2 plays a role in an inhibitory pathway for the induction of IL-17 and IL-22. These findings are in line with several studies that report an anti-inflammatory role for TLR2 through Th2 skewing (49, 50), the induction of anti-inflammatory cytokines by TLR2, and the TLR2-dependent induction of regulatory T-cells (51). Interestingly, TLR2 is a negative regulator of the Th17 response in a murine pulmonary infection model with *Paracoccidioides brasiliensis* (52), supporting a general role of TLR2 as a negative regulator of Th17 polarization of fungal infections.

The fact that the β-glucan receptor dectin-1 and its downstream kinase Syk play a redundant role in the induction of IL-17, IL-22, and IFNγ by hyphae, which abundantly express β-1,3-glucan, is rather unanticipated, since the dectin-1 pathway plays an important role in the host defence against invasive aspergillosis (35, 53). In line with our results, it has been demonstrated that IL-17 production by PBMCs with the dectin-1 Tyr238X polymorphism is not different from PBMCs that do not have this SNP (35). Notably, this SNP influences innate proinflammatory cytokines produced by PBMCs in response to *A. fumigatus* (35, 53). In mice, dectin-1 deficiency results in an increased susceptibility to *A. fumigatus* (3), that has been linked to disability to induce protective cytokines like IL-17 (3) and IL-22 (11). Although it is evident that dectin-1 plays a role in the induction of IL-17 and IL-22 in mice and that it plays a role in the innate host defence against invasive aspergillosis in humans, our studies suggest that IL-17, IL-22 and IFNγ production by $CD4^+$ T cells in the human host response to *Aspergillus* conidia is not predominantly mediated by dectin-1.

CR3 is a β2-integrin (CD11b/CD18) that is expressed by monocytes and neutrophils (54, 55). This receptor can recognize self-molecules such as complement, but it can also recognize PAMPs from pathogens such as LPS from *E. coli* (56). CR3 plays a role in phagocytosis and induction of cytokine responses (57). Here we show that CR3 is involved in modulating T-helper cytokine responses induced by *A. fumigatus*. To date, the modulation of Th17 and Th1 responses induced by fungi has not been linked to CR3 and future studies are required to further characterize its role in fungal infection. Interestingly, CR3 has been shown to bind b-glucan, which can have modulatory effects on the immune response, suggesting that fungal components can modulate proinflammatory T-helper responses (Th1 and Th17) through CR3 (58). Immunofluorescent staining of b-glucan revealed a very low expression on *Aspergillus* conidia, therefore b-glucan seems to be unlikely the main CR3 ligand in the recognition of *Aspergillus*.

In the present study we compared the IL-1β and IL-23 cytokine profiles produced by PBMCs stimulated with *A. fumigatus* and related this to the induction of IL-17 and IL-22. *Aspergillus* induced relatively low IL-17 levels, which was rather surprising since *A.*

fumigatus induced a significant number of IL-17 positive CD4 cells. These observations are in line with a previous report, which demonstrates that *A. fumigatus* is a poor inducer of IL-17 and is even capable of inhibiting the IL-17 response by interfering with the tryptophan metabolism (7). One possible explanation for the low IL-17 production could be that *Aspergillus* does not induce a significant IL-23 response, since IL-23 production was undetectable in *A. fumigatus* stimulations. Interestingly, supplementation of IL-23 to the *Aspergillus* stimulated PBMCs boosted the IL-17 response, which is in line with an earlier report which showed that IL-23 can augment IL-17 responses in *Aspergillus*-infected mice (3). Although, *Aspergillus* did not induce IL-23 in PBMCs, it was found earlier that DCs induce high levels of IL-23 upon stimulation with *Aspergillus* and that these DCs can polarize towards both Th1 and Th17 responses (37, 59). However, when we used monocyte-derived DCs instead of PBMCs in our experimental setup we were not able to detect any IL-23 or IL-1β in response to *Aspergillus* (data not shown). We further demonstrate that *Aspergillus*-induced IL-17 and IL-22 responses were dependent on IL–1. Notably, although IL-1β was present and the Th17 response was almost completely dependent on the IL–1 pathway, *Aspergillus* did not abundantly induce IL-17/IFNγ$^+$cells. This is in contrast with a previous study that suggests that IL-1β is responsible for the development of IL-17$^+$IFNγ$^+$ T-helper cells in response to the fungal pathogen *C. albicans*(44). These data provide evidence that next to IL–1, another pathway, such as the IL-23 pathway, must be triggered in order to induce IL-17$^+$IFNγ$^+$ cells.

Another striking observation is the role of TNF in modulating proinflammatory Th17 responses induced by *A. fumigatus*. Blocking TNF lowered the IL-17 production in response to *A. fumigatus*, moreover the effects were most prominent on IL-22 production. These observations are in line with previous reports, which demonstrated that IL-22 production can be triggered by TNFα (15, 60, 61), and is in line with the observation that anti-TNFα treatment can reduce IL-17 levels in patients(42). So far, the role of TNFα in pathogen-induced IL-22 production has not been described. This observation could be highly relevant given the widespread use of anti-TNF therapy. Notably, the use of anti-TNF (62-64) and polymorphisms in TNFR1 or decreased TNFR1 mRNA expression (64, 65) have been linked to increased susceptibility to invasive aspergillosis, underlining the importance to identify the impact of TNF blockade on anti-*Aspergillus* host responses, such as lowering IL-22 production in response to *A. fumigatus* as described in the present study.

In conclusion, we demonstrate that the T-helper cytokines IL-17, IL-22, and IFNγ in response to *A. fumigatus* are primarily produced by CD4 T-cells, and that these cytokines are not limited to a specific subset but can be produced by a variety of polarized T-helper subsets. TLR4 and CR3 and the cytokines IL-1, IL-23, and TNFα are of specific

importance for *Aspergillus*-induced IL-17 and IL-22, whereas we observed that the dectin-1/Syk pathway is redundant for this response. Collectively, these findings contribute to a better understanding of the human adaptive host defence against *A. fumigatus*, and provide new knowledge that contributes to the development of targeted adjunctive immunotherapeutic regiments in patients with invasive aspergillosis.

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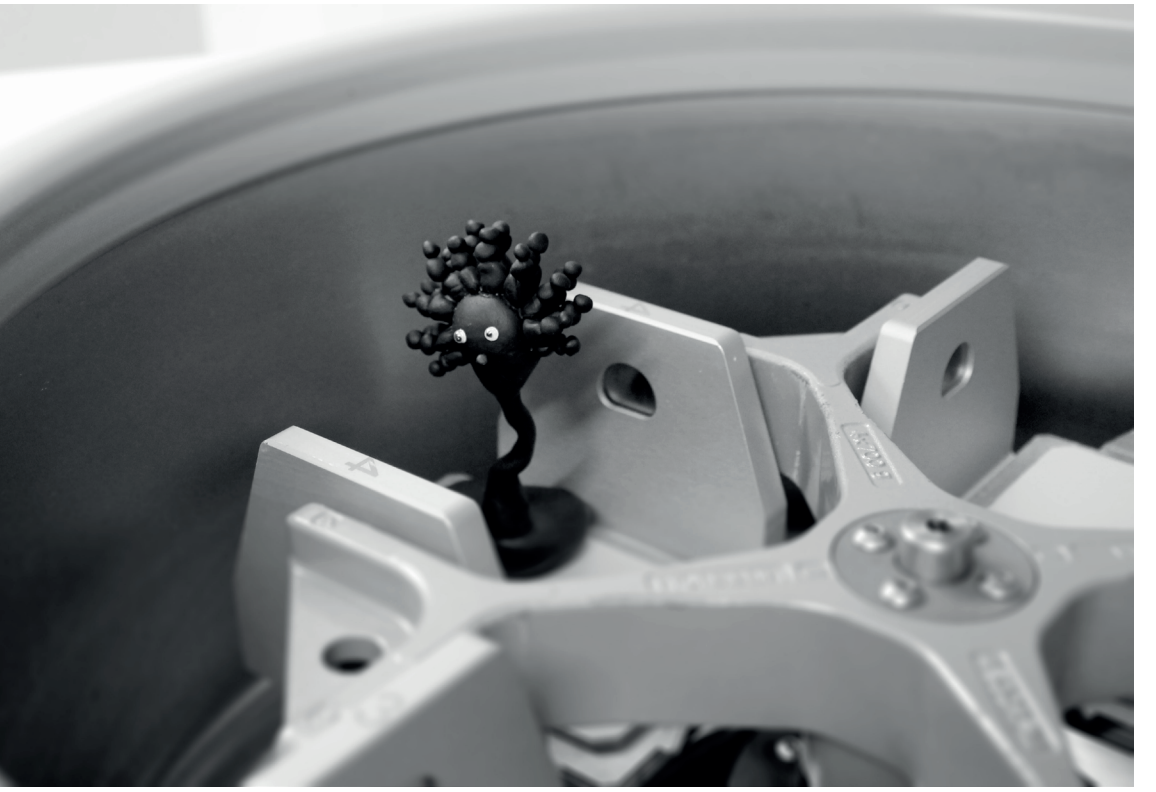

CHAPTER 5

Pattern recognition pathways leading to a Th2 cytokine bias in ABPA patients

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ABSTRACT

Background: Allergic bronchopulmonary aspergillosis (ABPA) is characterized by an exaggerated Th2 response to *Aspergillusfumigatus*, but the immunological pathways responsible for this effect are unknown.

Objective: The aim of this study was to decipher the pattern recognition receptors (PRRs) and cytokines involved in the *Aspergillus*-specific Th2 response, and to study *Aspergillus*-induced responses in healthy controls and ABPA patients.

Methods: Peripheral blood mononuclear cells (PBMCs) were stimulated with heat-killed *Aspergillus* conidia, various other pathogens, or PRR-ligands. PRRs and cytokine pathways were blocked with PRR-blocking reagents, anti-TNF (Etanercept or Adalimumab), IL-1Ra (Anakinra), or IFNγ (IFN-gamma). ELISA and FACS were used to analyze cytokine responses.

Results: *Aspergillus* was the only pathogen that stimulated the Th2 cytokines IL-5 and IL-13, while Gram-negative bacteria, Gram-positive bacteria, *Candida albicans,* chitin, b-glucan, or Toll like receptors (TLR) ligands did not. Depletion of $CD4^+$ cells abolished IL-13 production. Blocking complement receptor 3 (CR3) significantly reduced IL-5 and IL-13, while blocking TLR2, TLR4 or dectin-1 had no effect. ABPA patients displayed increased *Aspergillus*-induced IL-5 and IL-13, and decreased IFNγ production compared to healthy controls. All biological agents tested showed the capability to inhibit Th2 responses, but also decreased *Aspergillus*-induced IFNγ.

Conclusions: *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, through a CR3-dependent pathway. ABPA patients display a significantly increased *Aspergillus*-induced Th2/Th1-ratio that can be modulated by biologicals. These data provide a rationale to explore IFNγ therapy in ABPA as a corticosteroid-sparing treatment option, by dampening Th2 responses and supplementing the IFNγ-deficiency at the same time.

INTRODUCTION

Allergic Bronchopulmonary Aspergillosis (ABPA) is a hypersensitivity reaction against the ubiquitous mould *Aspergillus fumigatus* (1). Innate immune cells, such as lung epithelia cells or alveolar macrophages initiate this inflammatory reaction (2). Fungi are sensed by innate immune cells through pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), C-type-lectin receptors (CLRs) or nucleotide-binding oligomerization domain (NOD)-like-receptors (NLRs) (3). Activation of these receptors by *Aspergillus* leads to induction of cytokines, recruitment of other immune cells, and can eventually trigger adaptive immune responses such as T helper (Th) responses. A robust induction of the Th1 response during *Aspergillus* infection is associated with protection and successful clearance, while elevated Th2 responses impairs fungal clearance (4-6). Exaggerated Th2 responses are being held responsible for the detrimental inflammatory reaction observed in ABPA, namely eosinophilia, increased mucus production and immunoglobulin class switching to IgE. This inflammatory reaction subsequently results in airway hyper-responsiveness with typical asthmatic symptoms like bronchial obstruction, coughing and wheezing (7, 8). The current treatment for ABPA is the administration of oral corticosteroids during exacerbations and control of the underlying asthma with β_2-agonists. Furthermore, antifungal treatment with azoles in ABPA patients could spare the use of corticosteroids (1, 9). Due to the potential severe side effects of azoles and the long-term consequences of corticosteroid treatment, new approaches for ABPA therapy are needed.

While most *Aspergillus*-related PRRs have been studied in the context of invasive aspergillosis, limited data exists of the *Aspergillus*-associated molecular patterns and their PRRs responsible for triggering the Th2 response in ABPA. In mouse studies, TLR2 and TLR9 have been described to skew the *Aspergillus* induced Th response towards a Th2 profile (10, 11), and chitin exposure has been associated with lung eosinophilia (12). The current study aims to identify the immunological pathways through which *Aspergillus* induces a Th2 response in humans. Furthermore, the cytokine profile of a cohort of ABPA patients was investigated for assessing the Th1/Th2 balance. The capacity of treatment with biological therapy to modulate *Aspergillus*-induced Th-responses was tested *in vitro,* with the goal to find innovative corticosteroid-sparing treatment options for ABPA patients.

METHODS:

Volunteers and patients

Blood was collected from healthy volunteers or patients by venous blood puncture after informed consent. Three Dectin-1 deficient patients with a homozygous Y238X mutation in exon 6 of *CLEC7A* gene (the gene encoding Dectin-1) donated blood, two of them twice at two different time points. In these patients diminished expression of dectin-1 and failure to induce a cytokine response to β-glucan was demonstrated previously (13). Seven asthma patients and nine patients diagnosed with ABPA according to the ABPA diagnostic criteria as described elsewhere were asked for blood donations, two of whom had cystic fibrosis with a homozygous mutation in the *CFTR* gene (Table 1).

PRR ligands, blockers and other stimuli

E.coli LPS (10 ng/ml) (TLR4 ligand, *E. coli* serotype O55:B5, Sigma-Aldrich St. Louis, MO USA), Pam3Cys (10 μg/ml) (TLR2 ligand, EMC microcollections, Tübingen, Germany), Poly I:C (10 μg/ml) (TLR3 ligand, Cayla-Invivogen, Toulouse, France), flagellin (10 μg/ml) (TLR5 ligand, Cayla-Invivogen, Toulouse, France), N-acetylmuramyl-ananyl-D-isoglutamine (MDP) (10 μg/ml) (NOD-2 ligand, Sigma-Aldrich), phytohemagglutinin (PHA) (10 μg/ml) (Sigma-Aldrich). *Escherichia coli* ATCC 35218 (*E. coli*) ($1x10^7$ /ml); *Staphylococcus aureus* clinical isolate (*S. aureus*) ($1x10^7$ /ml); *Mycobacterium tuberculosis* sonicate H37Rv bub DL 6122005 (*M. tuberculosis*) (10 μg/ml); *Borrelia burgdorferi* ATCC 35210 (*B. burgdorferi*) ($1x10^6$ /ml); *Candida albicans* ATCC MYA-3573 (UC820) (*C. albicans*) ($1x10^6$ /ml); *Aspergillus fumigatus* clinical isolate V05-27 (*A. fumigatus*), resting conidia and hyphae ($1x10^7$ /ml,) were cultured and isolated as described in a previous study (14).

Fungal cell wall components: β1,3-(D)-glucan (β-glucan) (10 μg/ml) was kindly provided by Prof. David Williams (Tennessee University), and α1,3-glucan, α1,3-1,4-glucan (either dissolved in PBS or DMSO), chitin (*Aspergillus niger*) (all 10 μg/ml) and galactosaminogalactan (GAG) (7,5 μg/ml) were a gift from Prof. Jean-Paul Latgé (Pasteur Institute, Paris).

Bartonella quintana LPS was prepared and purified as described elsewhere (15) and used as a TLR4 inhibitor (20 ng/ml) (16); isotype control mouse IgG1 (10 μg/ml) (eBioscience, Halle-Zoersel, Belgium); anti-TLR2 (10 μg/ml) (eBioscience); laminarin (50 ng/ml) (17); a Syk-kinase inhibitor (50 nM, Merck, Darmstadt, Germany); isotype control goat IgG (10 μg/ml) (R&D Systems Minneapolis, MN, USA); anti-human β_2-integrin (anti-CR3) (10 μg/ml) (R&D Systems); anti-human OX40ligand (anti-OX40L) (10 μg/ml) (R&D Systems); cytochalasine D (1 μg/ml, dissolved in DMSO) (Enzo life sciences, Antwerpen, Belgium); IL-1 receptor antagonist (IL-1Ra) (10 μg/ml) (Amgen, Inc., Thousand Oaks, CA, USA); soluble TNFR2 (Etanercept) (100 μg/ml), human anti-human TNFα (Adalimumab),

Table 1: *Demographic table of ABPA patients*

No.	Sex	Age (y)	asthma/CF	total IgE (U/ml)	Aspergillus-specific IgG (mg/ml)	Aspergillus-specific IgE (U/ml)	eosinophils	Bronchiectasis	steroids use	Azole use
ABPA 1	F	22	dF508del/dF508del	60	48.60	1.5	1.06	yes	yes (ciclosenide, inhalative 160 mg)	yes
ABPA 2	M	21	dF508del/dF508del	229	40	13.9	0.79	yes	no	yes
ABPA 3	M	63	asthma	1406	353	25.1	2.44	yes	yes ((prednisone 5mg/d), 5mg/day)	no
ABPA 6	F	78	asthma	4515	n.d.	67.7	0.17	n/a	no	yes
ABPA 7	F	77	asthma	1263	120	46.4	0.88	Yes	no	no
ABPA 8	M	67	asthma	2710	128	29	0.88	Discrete	no	yes
ABPA 9	M	66	asthma	2508	187	15.1	n/a	Yes	yes (prednisolon, 10mg/d)	no
ABPA 10	F	70	asthma	3202	196	40.7	0.02	Discrete	yes (prednisolon, 15mg/d)	no
ABPA 11	M	77	asthma	3343	51	46.4	1.49	Yes	yes (prednison, 10mg/d)	no

Age, gender, underlying disease (asthma or cystic fibrosis), bronchiectasis, eosinophil count, total IgE (U/ml), *Aspergillus*-specific IgE (U/ml), *Aspergillus* -specific IgG (mg/ml) and concomitant medication (corticosteroids and azoles) of the ABPA patients enrolled in this study are listed.

(100 µg/ml), both kindly provided by Dr. Marije Koenders (Department of Rheumatology Radboud University Nijmegen Medical Centre the Netherlands); Interferon-gamma 1b (Immukine®) (50 ng/ml) (Boehringer-Ingelheim, Almaar, The Netherlands).

PBMCs isolation

Venous blood was drawn in 10 ml EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI-1640 culture medium (Dutch modification, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the concentration was adjusted to $1x10^7$ cells/ml.

Cell depletion

PBMCs were incubated with magnetic anti-$CD4^+$, anti-$CD56^+$ and anti-$CD19^+$ beads and purified using MACS depletion columns (LD, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions supplied by the manufacturer to generate $CD4^-$ (T-cells depleted), $CD56^-$ (NK-cells depleted) or $CD19^-$ (B-cells depleted) PBMC fractions. Next to the labeled cells, also an unlabeled PBMC fraction was run over the depletion column and used as a mock treatment control.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning, NY, USA) at a final concentration of $2.5x10^6$ /ml in an endvolume of 200 µl per well. All stimulations were performed in the presence of 10% human serum. Serum was either complement active if not otherwise indicated or heat-inactivated by incubation for 30 minutes at 56˚C in a water bath according to a commonly used protocol (18). Mannose-binding lectin (MBL) deficient serum was obtained from a patient with MBL level of 0.09 µg/ml. After 1h pre-incubation with inhibitor or medium, stimuli or medium were added. Cells were incubated at 37˚C with 5% CO_2, after 7 days supernatants were collected and stored at -20˚C.

Cytokines measurements

IL-5, IL-13, IFNγ and IL-10 were measured in the cell culture supernatants using a commercial ELISA kit (IL-5 and IL-13: R&D Systems; IFNγ and IL-10: Sanquin) according to the instructions supplied by the manufacturer.

Intracellular staining and flowcytometric analysis

After 7 days incubation, cells were re-stimulated for 4-6 hours with 200 µl of RPMI supplemented with gentamicin, L-glutamine and pyruvate and PMA (50 ng/ml) (Sigma-Aldrich), ionomycin (1 µg/ml) (Sigma-Aldrich), Golgiplug (BD Biosciences, Breda, the Netherlands) and 10% human serum. Cells were stained extracellular using PE-Cy7–conjugated anti-CD4 (BD Biosciences), PE-Cy7–conjugated anti-CD8 (BioLegend, San Diego), or PE-Cy7–conjugated anti-CD56 (Beckman Coulter) and ECD-conjugated monoclonal anti-CD3 antibody (Beckman Coulter, Krefeld, Germany). Fixation and permeabilization was performed with Cytofix/Cytoperm solution (eBioscience) according to the instructions supplied by the manufacturer. Cells were stained intracellular with anti-IL-4 (FITC conjugated), anti-IL-5 (PE conjugated) and anti-IL-13 (APC conjugated) (all BD Bioscience). Fixated cells were measured with a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

OX40L expression

RNA was isolated from $1x10^6$ PBMCs after stimulation for 48h with *Aspergillus* conidia either in the presence of medium or Adalimumab or IL-1Ra using Trizol Reagent (Invitrogen) according to a protocol supplied by the manufacturer. RNA (500 ng) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Hercules, Bio-Rad Laboratories, CA). Quantitative PCR (qPCR) analysis was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 real-time PCR system. As PCR protocol the following conditions were used: 2 min 50˚C, 10 min 95˚C followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min. For the amplification of hOX40L the primers 5'-TCCCCTCTCTTAGGTGCTCA-3' and 5'-GGCTGGTGCATAGCAGAAAT-3' were used. To correct for differences in loading concentrations of RNA between the different conditions, qPCR results were corrected with the housekeeping gene β2 microglobulin (β2m) amplified using the primers 5'- ATGAGTATGCCTGCCGTGTG-3' and 5'-CCAAATGCGGCATCTTCAAAC-3'. Primer efficacy was evaluated using a standard curve. OX40L Ct values were compared with the β2m Ct using the formula $2^{-\Delta Ct}$ and the foldchange was calculated by setting the *Aspergillus*-stimulated samples as 1 to determine the effect of anti-TNF (Adalimumab) and IL-1Ra on OX40L expression.

Genotyping

Genomic DNA from healthy caucasian controls was isolated using the Gentra Pure Gene blood kit (Qiagen, Hilden, Germany) according to the instructions supplied by the manufacturer. The specific SNP identifiers (rs numbers) were extracted from a recent publication describing SNPs associated with ABPA (19). Genotypes of the different SNPs were

obtained from the Illumina immunochip which has previously been run according to the manufactures protocol (20). Beforehand, a quality filter was applied which automatically excluded SNPs with either a low call rate (<99%), SNPs that were not in Hardy-Weinberg equilibrium (p<0.01) or that had a reported minor allele frequency of less than 0.01.

Statistical analysis

The Mann-Whitney-U test was used to detect differences between healthy controls and patients or between different genotypes. The Wilcoxon signed rank test was used to determine differences between stimulation with and without inhibitors of PRRs, cytokines or cytokine inhibitors. A *p*-value of < 0.05 was considered statistically significant ($^{*}=p < 0.05$, $^{**} = p < 0.01$ and $^{***} = p < 0.001$). Graphs represent cumulative results of all performed experiments and are presented as mean ± standard error of the mean. Data were analyzed with GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS:

Aspergillus conidia induce a Th2 response in human PBMCs, in contrast to other pathogens or PRR ligands

We investigated which PRRs are involved in the initiation of a Th2 response by stimulating PBMCs with different specific ligands for TLR4, TLR2, TLR5, NOD2 or Dectin-1, and measuring IL-5 and IL-13 (Figure 1A). None of these ligands or combinations of TLR2 with ligands for Dectin-1 or NOD2 was able to induce IL-5 or IL-13. We thereafter screened a panel of pathogens consisting of extra- and intracellular bacteria, as well as two types of fungi for their capacity to induce Th2 cytokines (Figure 1B). The whole bacteria *E. coli, S. aureus* or *B. burgdorferi,* as well as sonicated *M. tuberculosis* were unable to induce IL-5 and IL-13, while these pathogens induce other immune responses such as IL-17 and IFNγ in these conditions (data not shown). Among the fungal pathogens, the Th2 response was restricted to the stimulation with *A. fumigatus* conidia, while *C. albicans* yeast did not induce IL-5 and IL-13. Moreover, *Aspergillus* hyphae induced relatively low IL-5 production (76.4 pg/ml hyphae vs. 296 pg/ml conidia) and IL-13 (310 pg/ml hyphae vs. 568 pg/ml conidia). Stimulation with the fungal cell wall components α1,3-glucan and α1,3-1,6-glucan, β1,3-glucan, galactosaminagalactan (GAG) or chitin did not induce IL-5, while IL-13 production was slightly induced by α1,3-1,6-glucan or chitin (Figure 1C). PHA was used as a mitogenic stimulus to detect T cell memory activation, and induced low levels of Th2 cytokines.

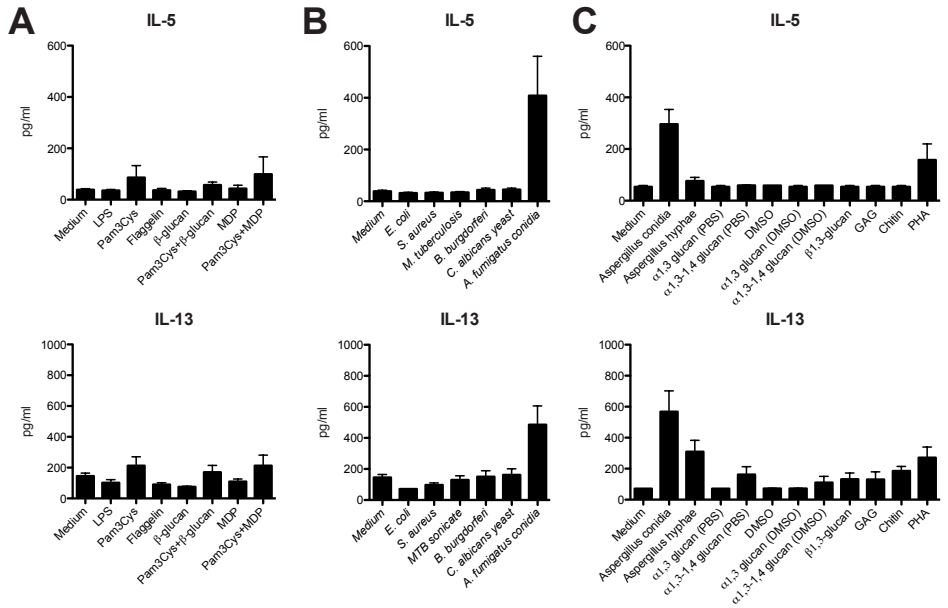

Figure 1: Aspergillus *conidia induce a Th2 response in human PBMCs in contrast to other pathogens or PRR ligands*

IL-5 and IL-13 concentrations were measured in cell culture supernatants of PBMCs of healthy controls (2.5 x 10^6 /ml, n=6 donors) by ELISA (IL-5 detection level 24 pg/ml, IL-13 detection level 71 pg/ml) after stimulation with (A) TLR4 ligand (LPS), TLR2 ligand (Pam3Cys), TLR3 ligand (Poly I:C), TLR 5 ligand (flaggelin), NOD3 ligand (MDP) or Dectin-1 ligand (β-glucan) (B) Various pathogens like *E.coli, S.aureus, M. tuberculosis* sonicate (H37Rv), *B. burgdorferi, C. albicans* yeast and *A. fumigatus* conidia (C) *A. fumigatus* conidia and hyphae as well as different cell wall components like α1,3 glucan, α1,3-1,4 glucan, β1,3 glucan, galactosaminogalactan, chitin PHA.

Aspergillus-induced IL-5 and IL-13 are dose and time dependent and predominantly produced by $CD4^+$ T cells

To study the kinetics and dose dependency of IL-5 and IL-13 induced by *Aspergillus* conidia, we performed a time course of 7 days with 3 different concentrations ranging from 1x10^5 to 1x10^7 conidia/ml (Figure 2A). IL-4 was undetectable in all stimulations (data not shown). In contrast, IL-5 and IL-13 were detectable from day 4 onwards and increased steadily. Only the highest dosage of 1x10^7 conidia/ml induced IL-5 and IL-13, while dosages below this threshold induced cytokine values barely detectable above the detection limit.

To investigate which cells are responsible for the production of IL-4, IL-5 and IL-13 induced by *Aspergillus*, flowcytometric analyses with intracellular staining for IL-4, IL-5

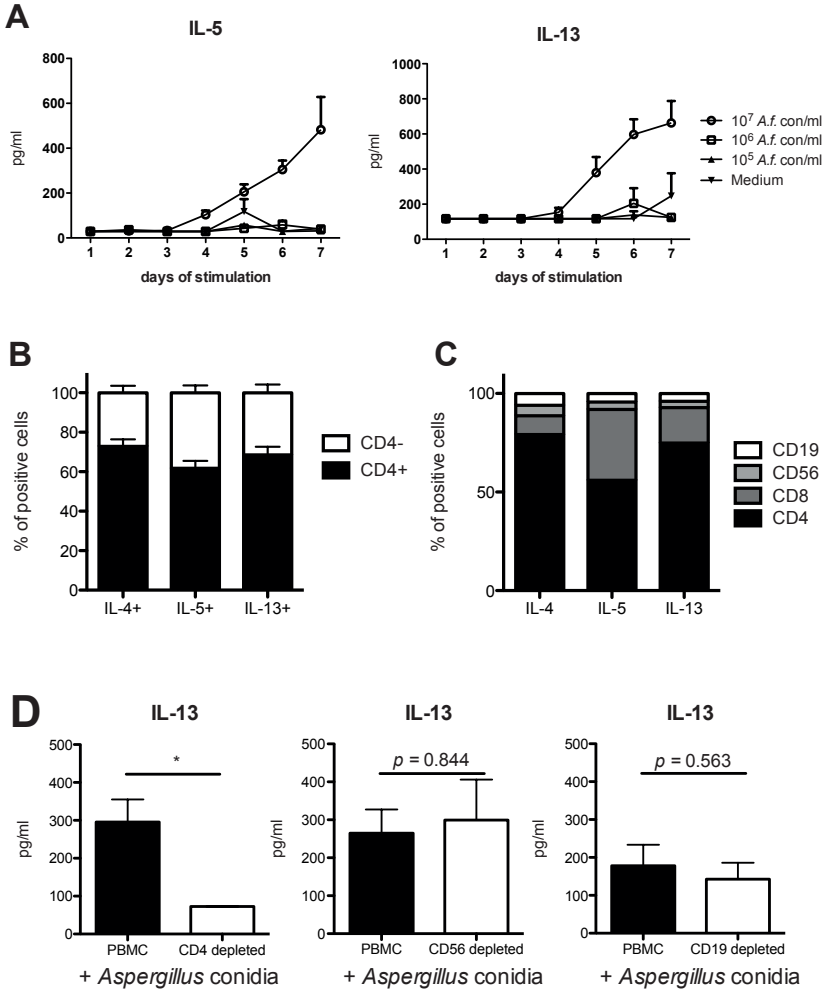

Figure 2: *Aspergillus-induced Th2 cytokines are dose and time dependent and predominantly produced by CD4⁺ cells*

(A) PBMCs (2.5 x 10^6 /ml, n=4 donors) were stimulated with increasing concentrations of *Aspergillus* conidia (1x10^5 /ml, 1x10^6 /ml, 1x10^7 /ml) for 1-7 days and IL-5 and IL-13 was measured in the cell culture supernatant by ELISA. (B-C) Cells were stained extracellular for CD4⁺-, CD8⁺-, CD56⁺ and CD19⁺ and intracellular for IL-4, IL-5 and IL-13. (B) CD4⁺ and CD4⁻ cells (n=10) within the IL-4, IL-5 or IL-13 positive cells. (C) CD4⁺-, CD8⁺-, CD56⁺ and CD19⁺-cells within the *Aspergillus*-specific IL-4⁺-, IL-5⁺- and IL-13⁺-populations (normalized to 100%, one representative donor) (D) PMBCs were depleted either for CD4⁺, CD56⁺ or CD19⁺ cells (white bars) or run over the depletion columns unlabeled (black bars) and stimulated with *Aspergillus* conidia. IL-13 was measured in the cell culture supernatant by ELISA (n=6 donors). The Wilcoxon Signed Rank test was used to determine, whether the means were significantly different (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

and IL-13 were performed on PBMCs that were cultured for 7 days in the presence of *Aspergillus* conidia. Within the Th2 cytokine positive population, $CD4^+$ cells were the main representatives (72.9% of $IL\text{-}4^+$, 61.7% of $IL\text{-}5^+$ and 68.5% of $IL\text{-}13^+$ cells) (Figure 2B). Surface staining for CD8, CD56 and CD19 revealed the $CD8^+$ cells as the second biggest subpopulation contributing to the IL-5 and IL-13 production (Figure 2C). To determine which cell populations within PBMCs contribute to the IL-5 and IL-13 secretion induced by *Aspergillus* stimulation, PBMCs were depleted of $CD4^+$, $CD56^+$ or $CD19^+$ cells (Figure 2D). Mock-treated PBMCs lost their capacity to produce IL-5, which made it impossible to evaluate the effect of depleting a special subpopulation on IL-5 production (Supplemental Figure 1). Depletion of the $CD4^+$ cells led to a reduction of *Aspergillus*-induced IL-13 production to undetectable levels, while depleting $CD56^+$ or $CD19^+$ cells did not affect IL-13 production. These data suggest that $CD4^+$ T-lymphocytes are the main producers of Th2 cytokines induced by *Aspergillus* conidia.

5

Aspergillus-induced IL-5 and IL-13 response is dependent on Complement Receptor 3 and phagocytosis

Since most PRRs have been studied in the context of invasive aspergillosis (3) only little is known about the PRRs and pathways involved in the pathogenesis of ABPA. We investigated the role of TLR2, TLR4, Dectin-1 and CR3 pathways in *Aspergillus*-induced Th2 responses in human PBMCs. PBMCs were stimulated with *Aspergillus* conidia in the absence or presence of specific inhibitors for the PRR pathways. Inhibition of TLR4 did not alter IL-5 production, while IL-13 was slightly reduced (mean 15.7% reduction, Figure 3A). Although TLR2 has been described to polarize *Aspergillus*-specific T cell responses towards Th2 (21), neither IL-5 nor IL-13 was affected when TLR2 was blocked (Figure 3B).

Dectin-1 is known to recognize the fungal cell wall component β-glucan (22) and is known to promote immunopathology in the lung during fungal allergy (23). However, blocking dectin-1 with laminarin (Figure 3C), or inhibition of Syk kinase, the downstream signaling kinase of the dectin-1 receptor, did not alter the Th2 response induced by *Aspergillus* (Figure 3D). Additionally, PBMCs isolated from three dectin-1 deficient patients produced IL-5 and IL-13 levels comparable to healthy controls (Figure 3E). Subsequently, we investigated CR3 (CD11b/CD18), a β_2-integrin expressed on neutrophils and monocytes, since it has been described that CR3 is also able to recognize β-glucan (24). Blockade of CR3 inhibited the *Aspergillus*-induced Th2 response completely (Figure 3F). We did not observe different cytokine production when the serum was de-complemented by heat-inactivation (Figure 3G).

Since CR3 is involved in phagocytosis (25), we blocked phagocytosis by inhibiting actin polymerisation by cytochalasine D, which significantly reduced IL-5 and IL-13 produc-

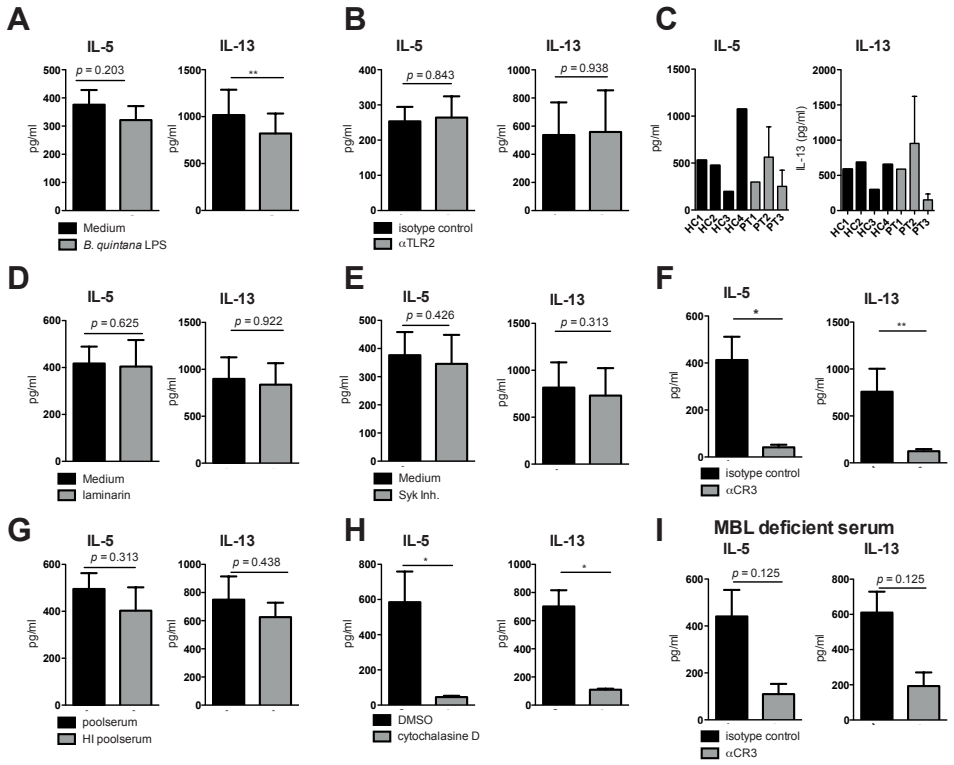

Figure 3: Aspergillus-*induced IL-5 and IL-13 response is dependent on Complement Receptor 3 and phagocytosis*

PBMCs were pre-incubated for 1h with different blockers and stimulated with *Aspergillus* conidia. IL-5 and IL-13 were measured in the cell culture supernatant (black bars: *Aspergillus* conidia stimulation with medium or control, grey bar: *Aspergillus* conidia stimulation after blocking). (A) TLR4 blocking with *B. quintana* LPS (IL-5 n=9 donors, IL-13 n=8 donors). (B) TLR2 blocking with αTLR2 antibody (IL-5 n=6 donors, IL-13 n=7 donors). (C) PBMCs of three Dectin-1 deficient patients compared with four healthy controls. (D) Dectin-1 blocking with laminarin (n=10 donors). (E) syk inhibition (IL-5 n=9 donors, IL-13 n=7 donors). (F) CR3 blocking with αCR3 antibody (IL-5 n=7 donors, IL-13 n=9 donors). (G) PBMC stimulation with 10% active human serum compared with 10% heat-inactivated human serum (IL-5 n=5 donors, IL-13 n=6 donors). (H) Blocking phagocytosis with cytochalasine D (n=6 donors). (I) CR3 blocking with αCR3 antibody (IL-5 and IL-13 n=4 donors) in the presence of MBL deficient serum. The Wilcoxon Signed Rank test was used to determine, whether the means were significantly different (*=p < 0.05, ** = p < 0.01 and *** = p < 0.001).

tion (Figure 3H). High plasma levels of MBL are associated with bronchial asthma with allergic rhinitis and ABPA (26), therefore we wanted to investigate whether the recognition of *Aspergillus* conidia with subsequent Th2 induction via CR3 is MBL dependent.

Aspergillus-specific Th2 cytokines in the presence of MBL deficient serum were significantly decreased after blocking CR3, suggesting that signaling via CR3 leading to Th2 responses is independent of MBL (Figure 3I). These data demonstrate that the IL-13 response induced by *Aspergillus* in human PBMCs is partially dependent on TLR4, while both IL-5 and IL-13 are dependent on CR3 and phagocytosis.

ABPA patients have an *Aspergillus*-specific increased Th2/Th1 ratio

PBMCs isolated from nine ABPA patients and healthy controls were stimulated with *Aspergillus* conidia and IL-5, IL-13 and IFNγ production was measured. Compared to non-allergic healthy controls, *ex vivo* induction of Th2 cytokines by *Aspergillus* stimulation was stronger in ABPA patients, which was statistically significant for IL-5 but not for IL-13 (Figure 4A). In contrast, *Aspergillus*-induced IFNγ production by PBMCs was significantly lower in ABPA patients when compared to healthy controls (Figure 4A). Interestingly, IFNγ was significantly decreased after stimulation with both *Aspergillus* conidia and hyphae (data only shown for conidia). When we compared the IL-5/IFNγ and IL-13/IFNγ ratios in ABPA patients and controls, we observed that they were significantly different and discriminated ABPA patients from healthy controls (Figure 4B, C).

To determine whether the elevated Th2/Th1 ratio in ABPA is due to the underlying asthma itself or due to *Aspergillus* sensitization, we investigated *Aspergillus*-specific ratios in asthma patients with and without an elevated *Aspergillus*-specific IgE (supplemental table 1). PBMCs isolated from asthma patients with *Aspergillus*-sensitization that were stimulated with *Aspergillus* conidia showed a higher *Aspergillus*-specific Th2 response than the asthma group without *Aspergillus*-sensitization and the control group (Figure 4D). This resulted in an elevated Th2/Th1 ratio comparable to ABPA patients (Figure E). In addition, we investigated whether the anti-inflammatory cytokine IL-10 is involved in the elevated ratios observed in ABPA. Neither in the control, nor in the patient group did we detect IL-10 concentrations (supplemental Figure 2).

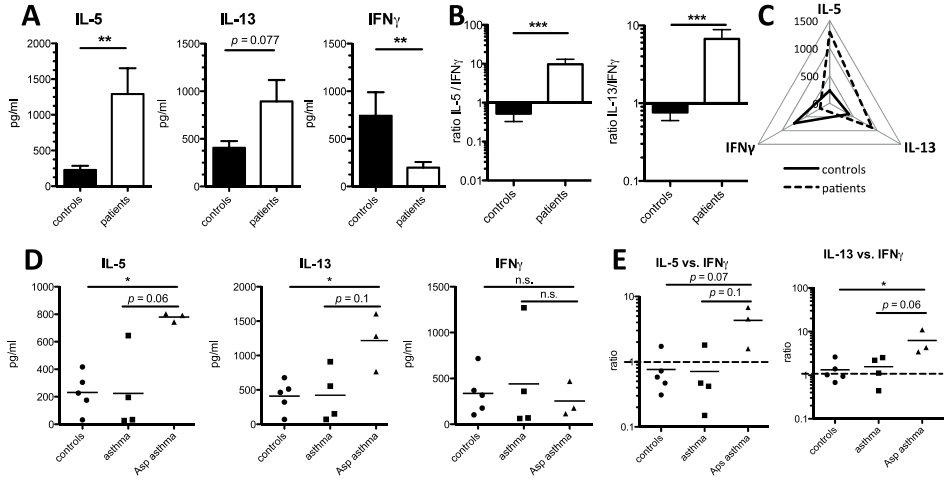

Figure 4: *The Th2/Th1 balance in ABPA patients is shifted towards Th2*

(A) PBMCs of 9 ABPA patients were stimulated with *Aspergillus* conidia and compared with PBMCs of healthy controls. IL-5, IL-13 and IFNγ levels were measured in the cell culture supernatant (black bars controls, white bars patients). (B) The Th2/Th1-ratio between IL-5 vs. IFNγ and IL-13 vs. IFNγ was generated by dividing the individual IL-5 or IL-13 level by the individual IFNγ level. (C) The triangle diagram shows the mean of IL-5, IL-13 or IFNγ of every group on the corresponding cytokine axis (continuous line: controls, dotted line: patients). (C) PBMCs isolated from 3 asthma patients with an elevated *Aspergillus*-specific IgE (Asp asthma) and 4 without elevated *Aspergillus*-specific IgE (asthma) were stimulated with *Aspergillus* conidia and compared with healthy controls. IL-5, IL-13 and IFNγ levels were measured in the cell culture supernatant. (D) The Th2/Th1-ratio between IL-5 vs. IFNγ and IL-13 vs. IFNγ was generated by dividing the individual IL-5 or IL-13 level by the individual IFNγ level. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=p < 0.05, ** = p < 0.01 and *** = p < 0.001).

Etanercept, Adalimumab, Anakinra and IFN-gamma have the capability to modulate the *Aspergillus*-induced Th2/Th1 ratio in human PBMCs

Since we observed significantly different Th2/Th1 ratios in ABPA patients we wanted to investigate whether available biologicals for treatment of inflammatory disease are capable of modulating the *Aspergillus*-specific Th2/Th1 ratio. We used Etanercept (soluble TNFα receptor II-Fc), Adalimumab (human anti-human TNFα), Anakinra (IL-1 receptor antagonist) and Immukine (recombinant human IFNγ) and stimulated PBMCs either with *Aspergillus* conidia in the presence of medium or one of the biologicals. All biologicals significantly suppressed *Aspergillus*-induced IL-5 and IL-13 production in human PBMCs. However, Etanercept, Adalimumab and Anakinra also suppressed IFNγ production in response to *Aspergillus* and hence did not correct the IL-5/IFNγ-ratios (Medium: 0.65,

Etanercept l: 0.61, Adalimumab: 0.92, Anakinra: 1.82) and IL-13/IFNγ-ratios (Medium: 1.49, Etanercept: 1.41, Adalimumab: 1.89, Anakinra: 2.77). Therefore, it was only recombinant IFNγ that was able to restore the increased Th1/Th2 ratio by down-regulating IL-5 and IL-13, but not IFNγ.

The co-stimulatory molecule OX40L is known to be involved in inflammatory Th2 responses triggered by thymic stromal lymphopoetin (TSLP)-activated dendritic cells in ABPA patients (27). We therefore investigated whether OX40L is also involved in the Th2 responses induced by *Aspergillus* in human PBMCs. Blocking OX40L resulted in significantly decreased *Aspergillus*-induced IL-13 production (FIG 5D). Since TNF and IL-1 can modulate the expression of OX40L (28, 29), we analyzed whether blocking TNF or IL-1 would result in a different expression of OX40L in PBMCs. We observed no significant modulation of the *Aspergillus*-induced OX40L expression in the presence of adalimumab or anakinra (Figure 5E).

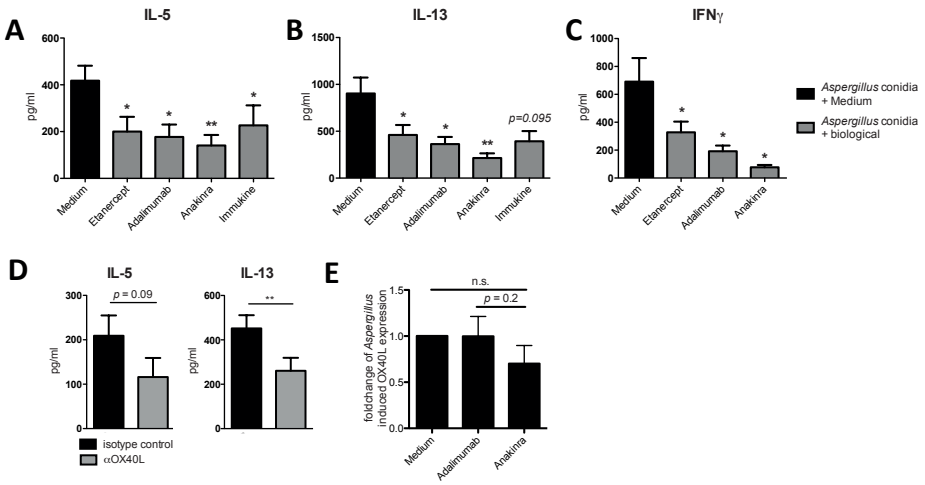

Figure 5: *Etanercept, Adalimumab, Anakinra and IFNγ have the capability to modulate the Aspergillus-induced Th2/Th1 ratio in human PBMCs*

PBMCs of healthy controls were stimulated with *Aspergillus* conidia either the presence of medium (black bars) or one of the biological drugs (grey bars) like Etanercept (soluble TNFα receptor), Adalimumab (human TNFα antibody), Anakinra (IL-1Ra) or recombinant IFNγ. (A) IL-5, (B) IL-13 and (C) IFNγ were measured with ELISA in the cell culture supernatant. (D) IL-5 and IL-13 measurement after blocking OX40L with αOX40L antibody (IL-5 and IL-13, n=9 donors). (E) OX40L expression in PBMCs of healthy controls stimulated with *Aspergillus* conidia either in the presence of Adalimumab or Anakinra (n=6). The Wilcoxon Signed Rank test was used to determine, whether the means were significantly different (*=p < 0.05, ** = p < 0.01 and *** = p < 0.001).

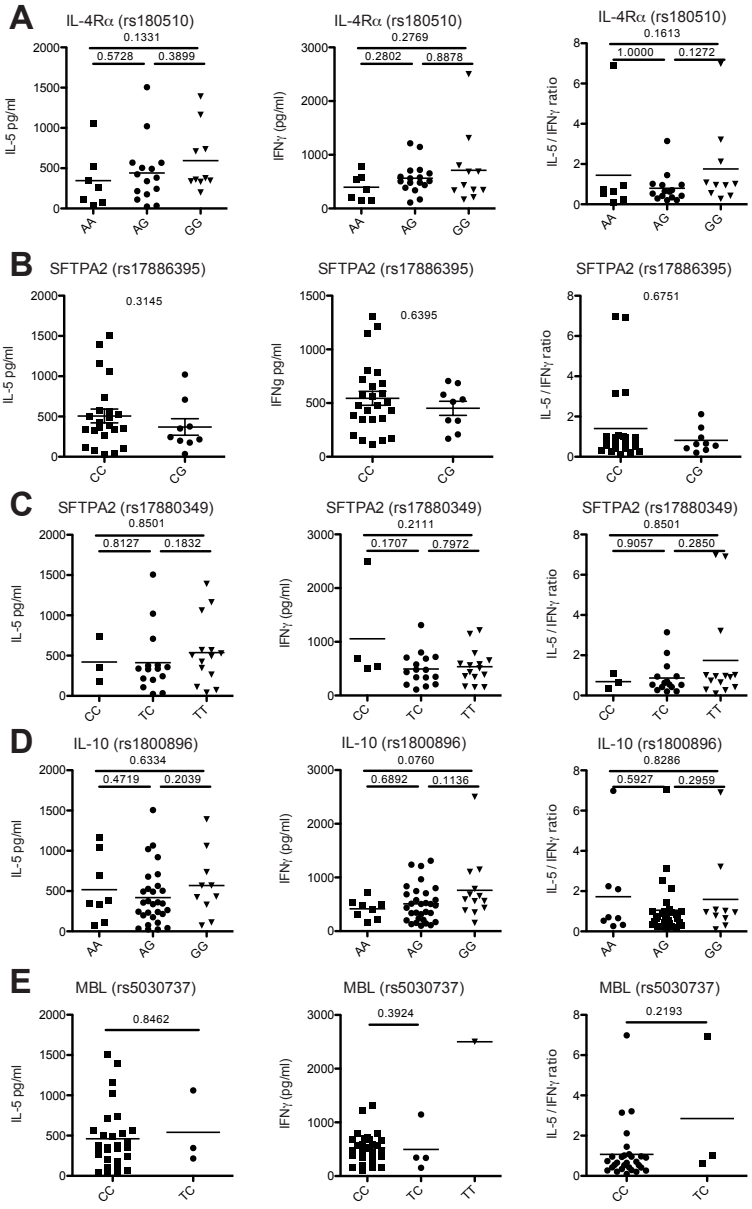

Figure 6: *The role of polymorphisms associated with ABPA on* Aspergillus*-specific Th2/Th1 ratios.*

PBMCs isolated from healthy volunteers (n=35) were stimulated with *Aspergillus* conidia and IL-5 and IFNγ were measured in the cell culture supernatant. SNPs were analyzed for association with cytokine production. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Polymorphisms influencing *Aspergillus*-specific Th2/Th1 ratios

Single nucleotide polymorphisms (SNPs) in the *IL10, IL4Rα, MBL*, and *SFTPA2*, gene have been associated with an increased susceptibility to ABPA (19). We took a systematic approach to investigate whether reported polymorphisms that are associated with ABPA could influence *Aspergillus*-specific Th2/Th1 ratios (table 2). PBMCs isolated from 35 healthy controls were stimulated with *Aspergillus* conidia and IL-5 and IFNγ was measured. The homozygote GG genotype of IL-4Rα (rs1805010) showed a trend towards higher IL-5 production and an increased IL-5/ IFNγ ratio (Figure 6A). The GG genotype in the IL-10 SNP (rs1800896) showed a trend towards higher IFNγ production, however no association was observed with *Aspergillus*-specific IL-5 or IL-5/ IFNγ ratio (Figure 6E). The other polymorphisms in SFTPA2 (rs17886395 and rs17880349) and MBL (rs5030737) did not show an association with IL-5 or IFNγ production (Figure 6B, C and D).

Table 2: *SNPs associated with ABPA*

Gene	rs number	SNP position	MAF	function
IL-4Rα (16p12.1- p11.2)	rs1805010	4679 A/G	0.466	missence
SFTPA2 (10q22.3)	rs17886395	1649 C/G	0.206	missence
SFTPA2	rs17880349	1492 C/T	0.461	Intronvariant
IL-10 (1q31-q32)	rs1800896	min 1082 A/G	0.303	near-Gene5
MBL (10q11.2-q21)	rs5030737	868 C/T	0.028	missence

Gene, rs number, SNP position, minor allele frequency (MAF) and resulting defect of gene function of SNPs associated with ABPA are listed.

DISCUSSION

In the present study, we investigated the general and *Aspergillus*-specific features of the Th2 response in primary human PBMCs of healthy controls and ABPA patients. Th2 cytokine responses *in vitro* could only be detected when PBMCs were stimulated with *A. fumigatus*, while *Candida,* specific PRR ligands, Gram-positive and Gram-negative bacteria did not induce IL-5 or IL-13 production. Furthermore, no single fungal component induced IL-5 or IL-13 in the same amount as *Aspergillus* conidia, suggesting that a complex of fungal PAMPs present on the cell wall of *Aspergillus* conidia is needed to induce a Th2 response in human PBMCs. The *Aspergillus*-specific IL-5 and IL-13 production was dependent on $CD4^+$ cells, the pattern recognition receptor CR3, and phagocytosis. Moreover, we identified that ABPA patients, in contrast to non-allergic controls, had a significantly elevated Th2/Th1 ratio induced by *Aspergillus*. Interestingly, not the asthma itself but the *Aspergillus* sensitization in asthma patients appears to be respon-

sible for the increased Th2/Th1 ratios observed in ABPA. We suggest that treatment strategies restoring this Th2/Th1 imbalance triggered by *Aspergillus* might be beneficial in the treatment of ABPA. Although anti-TNF and IL-1Ra had the capacity to decrease *Aspergillus*-induced IL-5 and IL-13 production they also lowered IFNγ production. Therefore, the capacity of recombinant IFNγ to lower IL-5 and IL-13 and to supplement the IFNγ deficiency in ABPA, provides a rationale to use IFNγ as a corticosteroid-sparing treatment option in ABPA.

The observation that the induction of Th2 cytokines in human PBMCs was restricted to *Aspergillus* was striking. In the context of diseases caused by bacteria such as *E. coli, S. aureus, M. tuberculosis, B. burgdorferi* it can be anticipated that they do not induce strong Th2 responses in human PBMCs. However, the observation that no single TLR ligand, NOD2 ligand, or fungal cell wall component, specifically chitin, did not induce IL-5 and IL-13 was unexpected. Chitin has been associated with allergic inflammation in the lung (30, 31). Therefore, it is most likely that chitin in the setting of an inflammatory milieu is able to polarize towards Th2 responses, while as a single ligand it is not able to induce a Th2 response.

This study revealed a non-predominant role of TLRs in the induction of *Aspergillus*-specific Th2 cytokines. Blocking of TLR2 did not have a significant impact on IL-5 and IL-13 production induced by *Aspergillus*. In contrast, it has been shown that there is an association between increased TLR2 expression in fatal asthma (32), and polymorphisms in TLR2 are associated with a higher prevalence of asthma (33). Furthermore, $TLR2^{-/-}$ mice show a deficient Th2 production in a chronic fungal asthma model (11). The minor but significant contribution of TLR4 in the *Aspergillus*-induced IL-13 induction found in the present study is in line with former studies describing TLR4 in allergic responses against house dust mite in which an endotoxin containing extract signaling via TLR4 (34). In addition, we identified a redundant role of dectin-1 in the *Aspergillus*-induced Th2 response. Blocking dectin-1 or Syk did not alter IL-5 and IL-13 production, and dectin-1 deficient patients produced Th2 cytokine responses comparable to healthy controls. The dectin-1 ligand β-glucan (17), however has recently been described to play an important role in *Aspergillus*-triggered asthma immunopathology in mice (23). The role of dectin-1 in the immunopathology of asthma is not clear. It has been suggested that dectin-1 promotes the immunopathology in fungal asthma (23), while another study demonstrated that dectin-1 plays a role in suppressing asthmatic inflammation (35). Our findings suggest that TLR2 and dectin-1 are not the main pathways driving the *Aspergillus*-specific Th2 response in human mononuclear cells. Whether TLR2 and dectin-1 play a role in *Aspergillus*-induced Th2 responses that are dependent on epithelial cells remains to be elucidated.

Th2 induction by *Aspergillus* was dependent on CR3 (CD11b/CD18). CR3 is a β_2-integrin that contains a lectin-like domain and is expressed on neutrophils and monocytes (25). CR3 contributes to antifungal host defense by interacting with pentraxin-3 opsonized particles, and by recognizing β-glucan(24). Furthermore, CR3 is involved in complement driven host responses (36), however the induction of IL-5 and IL-13 was still present in de-complemented serum, suggesting a complement independent contribution of CR3 to the Th2 response. Activation of the complement via the MBL pathway is an alternative route of the complement pathway, and increased MBL serum concentrations have been associated with ABPA (26). However, Th2 induction by *Aspergillus* via CR3 signaling was independent of serum MBL, suggesting that MBL is not crucial for CR3-mediated Th2 responses induced by *Aspergillus*. Phagocytosis of non-opsonized particles via signaling together with FcγR is also dependent on CR3 (25). In line with blocking CR3, blocking phagocytosis led to a complete abolishment of Th2 production induced by *Aspergillus*. These data collectively suggest that CR3 is involved in phagocytosis of *Aspergillus* conidia, and that this process is essential for Th2 cytokine production by human PBMCs. Recently, CR3 was described in the induction of the *Aspergillus* specific pro-inflammatory T helper cytokines (16), suggesting a more general role for CR3 in modulating T helper responses.

An important issue to address was the cellular source of the Th2 cytokine in human mononuclear cells induced by *Aspergillus*. We could demonstrate that $CD4^+$, but not $CD19^+$ and $CD56^+$ cells were the predominant source for the *Aspergillus*-induced IL-13 in human PBMCs. This is in line with previous studies showing that $CD4^+$ cells are responsible effector cells in allergic reactions, and that $CD19^+$ and $CD56^+$ play a redundant role in an asthmatic mouse model (37). Although increased levels of $CD8^+$ and IL-13^+ cells have been associated with asthma and bronchial obstruction (38), IL-13 production in human PBMCs was completely dependent on $CD4^+$ cells, suggesting that $CD8^+$ T cells are not involved in the production of *Aspergillus*-induced IL-13 in human mononuclear cells. We could not demonstrate the cellular source of IL-5 in our setting since IL-5 was not measurable in depletion experiments. Since FACS analysis demonstrated a significant $CD8^+$ population that stained positive for IL-5, we cannot exclude that $CD8^+$ T cells play a role in *Aspergillus*-induced IL-5 production in human PBMCs.

Several studies have shown an exaggerated Th2 response in ABPA (39-41)_ENREF_40. We investigated the *Aspergillus*-specific Th2 response in the context of *Aspergillus*-induced T helper responses in human PBMCs isolated from ABPA patients. Interestingly, ABPA patients not only show an increased IL-5 and IL-13 production after stimulation with *Aspergillus* conidia, which is in line with previous studies (42), they also have an *Aspergillus*-specific decreased IFNγ production. Since we also observed an increased

Th2/Th1 ratio in asthma patients with *Aspergillus*-sensitization, but not in patients with asthma that did not have *Aspergillus*-sensitization, it is most likely that the increased Th2/Th1 ratios in ABPA are due to an *Aspergillus*-specific host response and not due to the underlying host response associated with asthma itself. These observations strengthen the hypothesis that ABPA and asthma with *Aspergillus* sensitization share underlying pathophysiological mechanisms that are distinct from asthma itself, and support the concept that similar treatment approaches could be beneficial in both asthma-sensitized patients and ABPA, such as the treatment with azoles (9).

Aspergillus hyphae have been found in tissue biopsies in ABPA and have been suggested to play a significant role in inflammation in ABPA (43, 44). Furthermore, IgE antibodies directed against Aspf4 and Aspf6 can be observed in ABPA in patients with CF (45, 46). Aspf6 has been shown to be a hyphae-specific protein. Therefore, ABPA patients are exposed to *Aspergillus* hyphae, which contributes to a Th2 response in vivo. We observed however that *Aspergillus* hyphae induced much lower amounts of Th2 cytokines in ABPA patients compared to *Aspergillus* conidia, suggesting that fungal cell wall structures specific for *Aspergillus* conidia, or phagocytosis might play a more prominent role in the induction of Th2 responses in ABPA than previously thought.

Regulatory T cells (Tregs) are important for controlling allergic immune responses (47). A recent study has shown, that *Aspergillus*-sensitized asthma patients with high *Aspergillus*-specific Th2 induction had significant decreased levels of *Aspergillus*-specific Tregs, while they were strongly induced in the healthy control group (48). Also in CF patients with ABPA, Tregs were less frequent than in non-ABPA CF patients (27) suggesting that a deficient Treg response could contribute to the excessive and unbalanced inflammatory response observed in ABPA patients. It is tempting to speculate that the increased *Aspergillus*-specific Th2/Th1 response that we observed in the ABPA patients is due to a deficient Treg response, and that a deficient Tregs response, accompanied by a high Th2 and low Th1 response plays a significant role in the pathophysiology of ABPA.

The characterization of this immunological profile of ABPA patients with an elevated Th2/Th1 ratio provides a read-out for testing potential immunomodulatory treatment options in ABPA. TNF and IL-1 are interesting targets. TNF has been shown to be the key cytokine inducing increased IL-17 levels and neutrophilia in the lung in a murine ABPA model (49). The involvement of the inflammasome and epithelial derived IL-1 cytokines are also described in bronchial asthma in several studies (50, 51). Therefore we tested the TNF neutralizing biologicals, Etanercept and Adalimumab, and the IL–1 pathway blocking biological IL-1Ra (Anakinra). All biologicals decreased IL-5 and IL-13 production, but also IFNγ production, therefore resulting in unaltered IL-13/IFNγ and IL-5/IFNγ ratios. In

addition we investigated recombinant IFNγ, which decreased *Aspergillus*-specific IL-5 and IL-13 production.

The co-stimulatory molecule OX40L is involved in the initiation and maintenance of allergic responses, mainly driven by TSLP activated dendritic cells (52). Clinical studies that have been performed to elucidate the role of OX40L as a possible therapeutic target in asthma and have shown that blocking OX40L results in a decrease of serum IgE and eosinophils in asthma (53). OX40L was also involved in the *Aspergillus*-induced Th2 response, which is in line with former studies showing OX40L expressed on a variety of cells like activated T cells, NK cells, B cells and monocytes (54). We observed a low expression of OX40L in PBMCs that was not significantly modulated by TNF or IL-1. Although it is reported that TNF can increase OX40L expression in smooth muscle cells, significantly higher in asthmatic than non-asthmatic patients (28) and TNF can upregulate OX40L promoter-activity (29), this mechanism appears to play a more prominent role in DC-driven Th2 responses (55).

Taking the shifted *Aspergillus*-induced Th1/Th2 ratio into account, restoring the increased Th1/Th2-ratio might be an interesting alternative for target-specific immunomodulatory therapy in ABPA. Previous studies have shown that neutralizing IL-13 has beneficial effects in an asthmatic mouse model (56) by dampening the IL-13 driven allergic airway inflammation. Similarly, blocking IL-4 has been tried to attenuate allergic airway responsiveness, however the effect was not as strong and long-lasting as blocking IL-13(56). Biologicals targeting IL-4, IL-5 and IL-13 have been shown to have some efficacy in patients with high Th2 cytokine levels, however optimal biomarkers to identify responders and non-responders are still lacking (57). There is only little clinical evidence for immunomodulatory treatment with biologicals in ABPA. Omalizumab has been successfully used in a few cases of ABPA with and without CF resulting in a corticosteroid-sparing treatment (58-60). The data of this study suggests that IFNγ might be an adequate immunomodulatory therapeutic option for ABPA, since it has the capacity to restore the increased Th1/Th2-ratio. Next to lowering the Th2 response it also supplements the relative *Aspergillus*-specific IFNγ deficiency. Recombinant IFNγ has been used with success in patients with chronic granulomatous disease to prevent *Aspergillus* infection (61) and there are several case reports that describe the beneficial effect of IFNγ treatment in *Aspergillus* infection (62). Thus, IFNγ might contribute to disease control in ABPA by having a beneficial effect on clearing the fungal burden thereby decreasing the trigger of the allergic inflammatory reaction, and by restoring the increased Th2/Th1 ratio.

Single nucleotide polymorphisms (SNPs) in the *IL10, IL4R*α, *MBL*, and *SFTPA2* genes have been associated with an increased susceptibility to ABPA (19). We therefore in-

vestigated whether these genetic variants could modulate the *Aspergillus*-induced Th1 or Th2 response. Variants in the MBL (63) and SFTPA2 (64) genes did not lead to different *Aspergillus*-specific Th2 and Th1 responses. In contrast, we could identify a trend between the IL-4Rα (65) GG genotype with higher *Aspergillus*-specific IL-5 production and *Aspergillus*-specific Th2/Th1 ratio. We observed a trend in the IL-10 (rs1800896) GG genotype towards higher IFNγ production. Previous studies have identified this genotype to be associated with *Aspergillus* colonization and ABPA in CF patients (66).

In conclusion, *Aspergillus* conidia are unique in triggering Th2 responses in human PBMCs, and we provide evidence that CR3 and phagocytosis play a predominant role in *Aspergillus*-induced Th2 responses. Moreover, we identified that patients with ABPA have an increased *Aspergillus*-specific Th2/Th1 ratio, and the data in this study together with the clinical experience and safety of IFNγ treatment provide a rationale for exploring IFNγ in a clinical trail in patients with ABPA.

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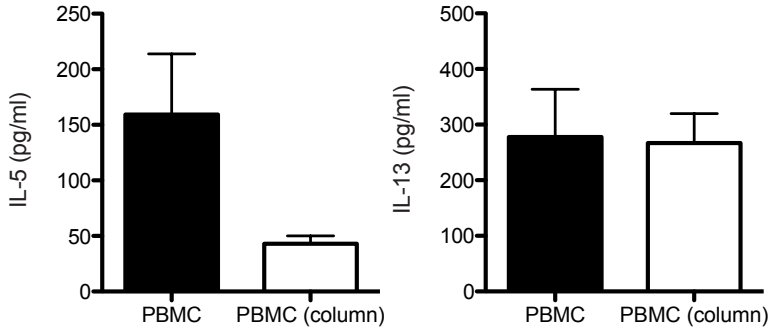
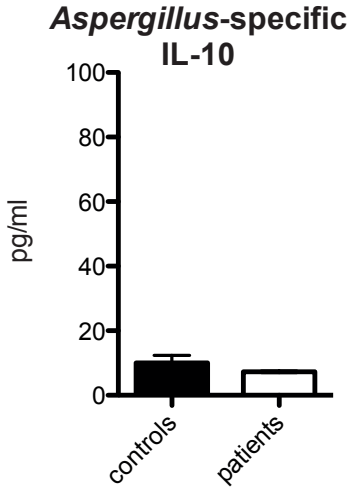


Supplemental Figure 1: *The Th2 response against* Aspergillus *conidia of PBMCs before and after running over a cell depletion column.*

PBMCs were either directly stimulated or mock treated by running unlabeled cells of a cell depletion column. PBMCs (2.5 x 10^6 /ml, n=4 donors) were stimulated with *Aspergillus* conidia and IL-5 and IL-13 were measured in the cell culture supernatant by ELISA.



Supplemental Figure 2: *IL-10 induction by* Aspergillus *conidia of PBMCs in ABPA patients and healthy controls.*

PBMCs (n=9 ABPA patients or healthy controls) were stimulated with *Aspergillus* conidia for 7 days and IL-10 was measured in the cell culture supernatant by ELISA.

Supplemental table 1: *Demographic table of asthma patients*

	age	gender	Asp. Spec. IgE (U/ml)	Asp. Spec. IgG (mg/ml)	total IgE (U/ml)
Asp-asthma #1	61	m	>5	neg	1913
Asp-asthma #2	76	m	11,2	62,1	570
Asp-asthma #3	44	m	>5	3,76	52
asthma #1	44	f	<0,35	n.a.	79
asthma #2	23	f	<0,35	36,2	212
asthma #3	47	f	<0,35	4,11	182
asthma #4	47	f	<0,35	43,1	95

Age, gender, total IgE (U/ml), Asp-specific IgE (U/ml) and Asp-specific IgG (mg/ml) of the asthmatic control groups enrolled in this study are listed.


OAP
ETHANOL
70 %
Gefahr / Danger
SOAP

CHAPTER 6

Deciphering the pathophysiology in patients with Allergic Bronchopulmonary Aspergillosis using a combined immunological and genetic approach

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Manuscript in preparation (Letter)

LETTER TO THE EDITOR

Allergic Bronchopulmonary Aspergillosis (ABPA) is a hypersensitivity reaction to the common mould *Aspergillus fumigatus* in the lung (1, 2). It occurs predominantly in patients with asthma and cystic fibrosis. Interestingly, it has a high prevalence in India. While Non-Indian asthma patients suffer in 3.3% of the cases from ABPA, these are 16% of all asthma patients in India (3). Because a recent study did not find environmental factors to have a strong influence on the prevalence of ABPA, it has been suggested that the genetic background is the main cause driving pathophysiology in ABPA (4). Clinically, ABPA patients present with typical asthmatic symptoms such as coughing, wheezing and shortness of breath, but the exact immunopathological processes causing these clinical symptoms are not yet fully understood.

After inhalation of the *Aspergillus* conidia an innate immune response gets initiated: bronchial epithelial cells, alveolar macrophages, as well as neutrophilic and eosinophilic granulocytes contribute to the innate host defence against *Aspergillus* conidia. Epithelial cells internalize *Aspergillus* conidia into acid organelles, through which the conidia succeed entering the body (5, 6). Additionally, *Aspergillus* proteases and proteins lead to damage of the epithelial barrier and activate macrophages resulting in secretion of a broad panel of pro-inflammatory cytokines such as IRF8, IL-1β and IL-6 and chemokines (7). Neutrophil recruitment was shown to be essential for effective control of the *Aspergillus* conidia (8), but pathologically characteristic for allergic reactions in ABPA is an overwhelming eosinophilic influx (9). This promotes further damage of the bronchial epithelium (10) leading to decreased barrier function maintaining the hypersensitivity reaction.

After recognition by the innate immune system, adaptive T helper responses are initiated. The balance between the different T helper subsets is crucial for the *Aspergillus*-specific immune response. TNFα derived from dendritic cells was shown to play a regulatory role in the activation of Th17 cells during an experimental ABPA model, promoting neutrophilic airway inflammation rather than Th2 cells activation, which leads to reduced eosinophil recruitment (11). While a robust Th1 response, reflected by a high IFNγ production, was associated with the successful clearance of the fungus, ABPA patients are described to have an exaggerated Th2 response and decreased Th1 response (12). As a result, they fail to eliminate the fungus efficiently, predisposing the host to allergic reactions (13). Whether Th17 cells play a protective or detrimental role in the allergic pathology is still unclear.

Treatment can be targeted at the host immune response level by dampening the hyper-reactive immune system with systemic corticosteroids, or targeted at the pathogen level with antifungal drugs (14). However, long-term treatment with corticosteroids

and itraconazole was associated with severe side-effects (15). Therefore, a fundamental understanding of the immunopathological processes underlying ABPA is needed to find more target-specific and effective treatment options in APBA. In this study we used a systems biology approach by comparing more than 120,000 genetic polymorphisms in ABPA patients, asthma patients and healthy controls, and we integrated genetic and functional data to define the pathways important for ABPA.

I. GENETIC ASSOCIATIONS COMPARING ABPA PATIENTS AND HEALTHY CONTROLS

We collected blood from 98 Indian ABPA patients and 95 Indian asthma patients for genetic association. Blood from 852 Indian healthy controls was used from a previous study. After DNA isolation 124,784 genetic markers were measured by genotyping using a custom-made Immunochip according to Illumina's protocols (16), and subsequently analysed using logistic regression including gender and three principal components as covariants. We applied SNP quality-control filters to exclude single nucleotide polymorphisms (SNPs) with (a) a low call rate (<99%), (b) a Hardy–Weinberg equilibrium of P<0.01 in control samples only and (c) a minor allele frequency (MAF) of < 0.01. We excluded 18 samples in the ABPA cohort, 97 samples in the control cohort and 9 asthma patients

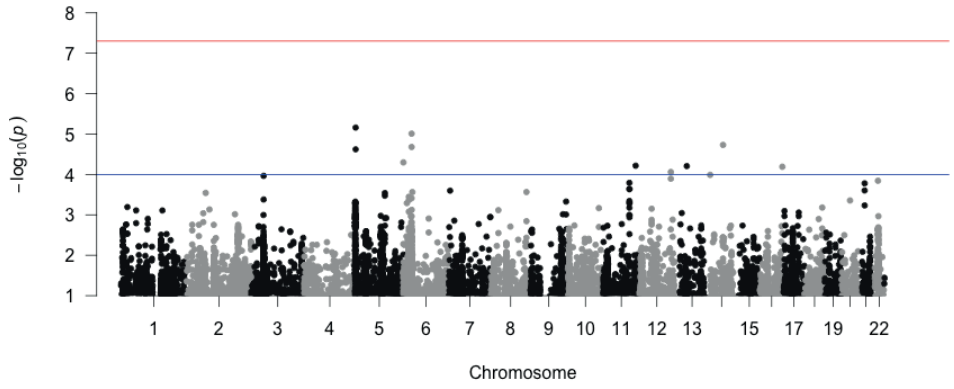

Figure 1: *Manhattan plot with 10 suggestive associations with a p value of < 9.9 x 10^{-4}*

Manhattan plot showing the P values of the genes associated with ABPA resulting from the Immunochip-assay. The y-axis represents the -log10 P values of 118,989 SNPs and their chromosomal positions are shown on the x-axis. The horizontal line shows the genome-wide significant threshold of P<9x10^{-4}. The P-values were obtained by logistic regression test after adjusting for the first three components from the multidimensional scaling analysis.

because of duplicates (n=21), relationship (n=43) or a low genotyping rate (<98%) (n=45). Genetic matching between cases and controls was tested based on multidimensional scaling (MDS) analysis.

We tested association at 118,989 SNPs between ABPA and healthy controls using logistic regression test by including age, sex and four MDS components as covariates. The results of this analysis did not identify genome-wide significant ($P < 5 \times 10^{-8}$) associations. However, we considered a threshold of $P<9.9\times10^{-4}$ as significant association to identify underlying susceptibility genes and discovered 10 independent loci that showed suggestive association with ABPA (Figure 1, Table 1). The comparison of ABPA patients with the asthmatic controls was not significant. We subsequently performed *cis*-eQTL mapping at the ten suggestive loci and revealed five SNPs (rs3130559, rs6898137, rs1745836, rs6573859, rs62051232) that significantly correlated with the expression of several genes that could be categorized into three relevant pathways for ABPA, namely the IRF8, the STAT3 and the vitamin D receptor pathways.

Table 1: *Ten Suggestive genetic associations*

CHR	SNP	OR	STAT	P
3	rs34622981	2.428	4.117	3.84E-05
3	rs7630157	2.796	4.364	1.28E-05
4	rs10015016	7.587	3.955	7.64E-05
5	rs6898137	2.759	4.78	1.76E-06
6	rs9405643	2.385	3.905	9.41E-05
6	rs3130559	2.251	4.263	2.02E-05
13	rs1745836	2.719	4.45	8.58E-06
14	rs6573859	2.174	4.332	1.48E-05
16	rs62051232	2.1	4.03	5.58E-05
18	rs17663691	5.928	4.504	6.67E-06

II. FUNCTIONAL VALIDATION OF THE GENETIC HITS

a. IRF8

The polymorphism rs62051232 was significantly associated with the regulation of the gene IRF8 resulting from eQTL mapping. IRF family proteins bind to the IFN-stimulated response element (ISRE) and regulate expression of genes stimulated by type I IFNs. Also *Aspergillus* has been described to induce type I interferons in respiratory epithelial cells *in vitro* (17), and IFNβ led to expression of IFNβ inducible genes, such as the IFNγ inducible protein IP10. For the functional validation, serum samples of 62 ABPA patients were

collected and cytokines derived from various cell populations were analysed. Comparing the serum IP-10 level of ABPA patients and healthy controls from the same endemic area revealed no significant differences between both groups, while IL-12 and IFNγ were significantly higher in the ABPA patients (Figure 2). Interestingly, IFNγ enhances the IRF8 expression in macrophages (18). The predominant Th1 response was an unexpected finding in ABPA, since a predominant Th2 helper with low Th1 responses has been demonstrated in other studies (12, 19, 20). In addition to IFNγ, IL-4 was significantly higher in ABPA patients' sera (Figure 2). To better understand the functional role of the cytokine profile of ABPA serum, a bioactivity assay was performed. PBMCs isolated from healthy volunteers were stimulated with *Aspergillus* conidia in the presence of ABPA serum or healthy control serum. Interestingly, ABPA serum skewed the immune response towards a pro-inflammatory profile. IL-6 was significantly higher, while IL-5 was significantly lower in the presence of ABPA serum (data not shown). Other studies have also investigated pro-inflammatory cytokines in ABPA, with Walker *et al.* describing elevated IL-6, TNFα

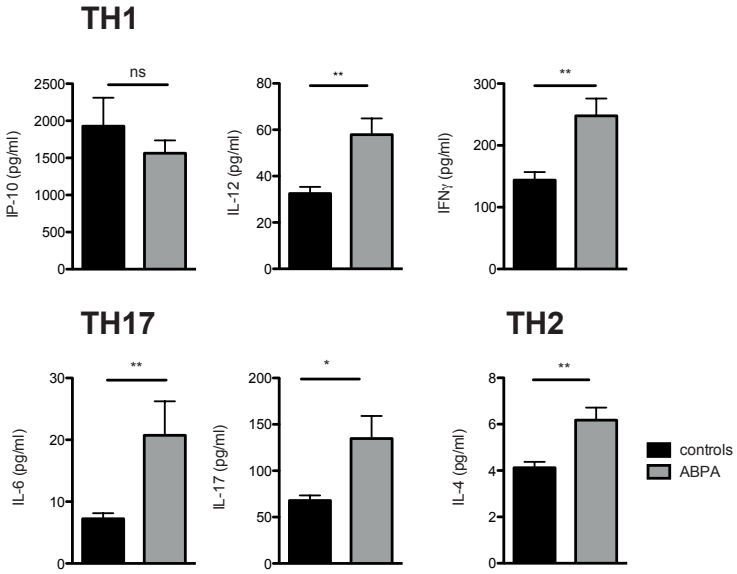

Figure 2: *ABPA patients have elevated T helper responses compared to healthy controls,*

Cytokines derived from different cell types were measured in treatment naïve ABPA serum (n=14) or control serum (n=12). IP-10, IL-12 and IFNγ were measured representative for the Th1 response. IL-6 and IL-17 for the Th17 response and IL-4 for the Th2 response in the serum using Luminex magnetic beads ELISA. Statistical analysis was performed with the Mann-Whitney test.

and IL-1β levels in BAL fluid of ABPA patients compared to healthy controls that is in line with the elevated circulating pro-inflammatory cytokine levels in the present study (21).

b. STAT3 pathway

The second striking result from eQTL mapping for ABPA associated SNPs was predominance of genes involved in STAT3 regulation. rs3130559 was significantly associated with the regulation of seven different genes, namely TCF19, CCHCR1, HCG27, POU5F1, HCG22, HLA-C and rs1745836 with the regulation of LRCH1 and ESD. Interestingly, œ genes had a transcription factor-binding site for STAT3. STAT3 is induced by IL-6, IL-1β or IL-23 and drives the differentiation of a naïve T helper cell towards a Th17 cells (22). To functionally validate the role of STAT3 in *Aspergillus* induced cytokine responses, we stimulated PBMCs of STAT3 deficient patients with *Aspergillus* conidia and measured IL-17 and IL-22. All patients showed deficient production of Th17 derived cytokines after stimulation with the fungus (Figure 3A). This suggests that STAT3 is involved in

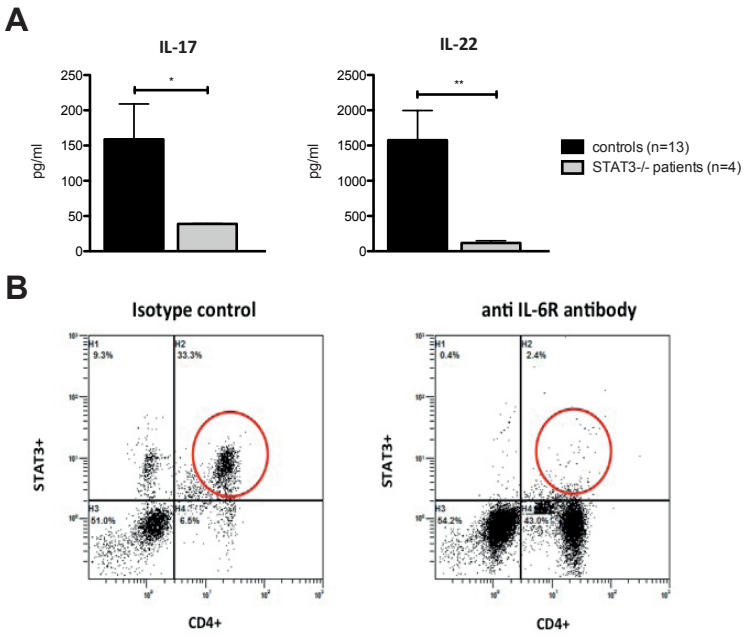

Figure 3: *STAT3 activation by Aspergillus is dependent on IL-6 pathway*

(A) PBMCs of healthy controls and HIES patients with a STAT3 loss of function mutation were stimulated with *Aspergillus* and Candida. IL-17 and IL-22 were measured in the cell culture supernatant by ELISA. (B) PBMCs of healthy volunteers were stimulated with *Aspergillus* and STAT3 activation on CD4+ cells was measured in the presence of an isotype control and anti-IL-6 receptor antibody.

the *Aspergillus*-specific Th17-response. As mentioned above we observed that the net cytokine profile in the serum skews *Aspergillus*-induced responses towards increased IL-6 production. To confirm the role of IL-6 in the *Aspergillus*-specific STAT3 induction, we stimulated PBMCs with *Aspergillus* and measured the frequency of STAT3 positive CD4+ cells after blocking the IL-6 receptor: in this experimental setting STAT3 activation was reduced (Figure 3B). This underscores the role of IL-6 in the *Aspergillus*-induced STAT3 activation. Subsequently, we analyzed IL-6 and IL-17 serum concentrations of ABPA patients. Both cytokines were significantly higher compared to healthy controls (Figure 2). Therefore, next to the elevated Th1 and Th2 response, ABPA seems to be characterized by an elevated Th17 response that might be dependent on IL-6 and a genetic predisposition for increased STAT3 function.

The STAT3 activation by *Aspergillus* has been suggested by former studies as well. Chen *et al.* showed by analysis of the transcriptome of *Aspergillus*-stimulated A549 cells that the STAT3 pathway is significantly upregulated (23). Also the functional role of STAT3 has been investigated in a mouse model of multi-allergen induced asthma. STAT3 inhibition had the same effect as the administration of corticosteroids on to the inflammatory cell influx seen by the histological analysis as well as by the dampening effect on the IL-17 response (24). These data are suggestive of an increased STAT3 pathway in ABPA and might be promising for a more target-specific treatment option in ABPA.

c. Vitamin D receptor

The SNP rs4334089 was significantly associated with the expression of the vitamin D receptor in a cis-eQTL analysis of peripheral blood RNA–sequencing data from 2,116 unrelated individuals as described by Zhernakova *et al.* (25). To elucidate the functional relevance of the influence of vitamin D on the T helper cytokine production induced by *Aspergillus,* we performed experiments in the presence or absence of vitamin D *in vitro*. Vitamin D had an inhibitory effect on all T helper subsets induced by *Aspergillus* (Figure 4). Recently, the role of vitamin D as a treatment option has been explored in patients with ABPA. Kreindler *et al.* observed the attenuation of the *Aspergillus*-specific Th2 response of CF patients with ABPA by vitamin D *in vitro* (26). Additionally, a recent clinical trial confirmed this data: *Aspergillus*-specific Th2 responses of ABPA patients were decreased after supplementation of vitamin D (27).

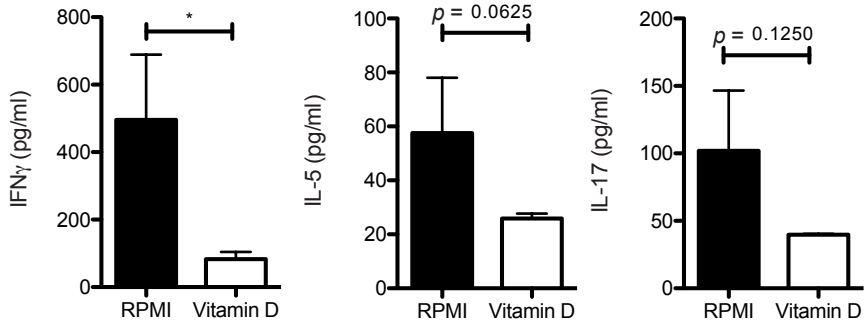

Figure 4: *T helper responses are dampened in the presence of Vitamin D*

PBMC of healthy volunteers were stimulated with *Aspergillus* conidia in the presence of RPMI or vitamin D. The cytokines IFNγ, IL-5 and IL-17 were measured in the cell culture supernatant by ELISA.

Collectively, these findings reveal novel insights into the pathophysiology of ABPA. By using a systems biology approach we identified three interesting pathways that might prove to be important in the pathophysiology of ABPA and could help to design novel treatment strategies. Supplementation with vitamin D resulted in a better clinical outcome of ABPA patients with CF as recently published in a clinical trial. The polymorphism in the vitamin D receptor gene described in this study supports this data and proposes a beneficial effect of vitamin D on the clinical presentation of Indian ABPA patients as well. ABPA patients had polymorphisms in genes associated with the Th1 and Th17 pathway, suggesting that the pathophysiology of ABPA might be dependent on a dysregulated Th1 and Th17 response, in addition to the well-known exaggerated Th2 response. We found pro-inflammatory and Th17 derived cytokines in the ABPA serum as well as polymorphisms in genes that were predominantly regulated by STAT3. This might open strategies to block these inflammatory pathways in ABPA: such as treatment with anakinra, (interleukin-1 receptor antagonist) that has the capacity to block STAT3-induced Th17 responses (28) or anti-IL-6 therapy (tocilizumab) that targets IL-6 that drives STAT3-dependent Th17 responses. Novel treatment options are urgently needed for ABPA and research validating the pathways identified by genetic studies is the next step to identify to provide the rationale to explore experimental options in ABPA.

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CHAPTER 7

Differential kinetics of *A. nidulans* and *A. fumigatus* phagocytosis

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SUMMARY:

Invasive aspergillosis mainly occurs in severely immunocompromised patients and is commonly caused by *Aspergillus fumigatus,* while *Aspergillus nidulans* is rarely found. By contrast, in chronic granulomatous disease (CGD) patients, *A. nidulans* is a frequent cause of aspergillosis and is associated with higher mortality. Immune recognition of *A. nidulans* was compared to *A. fumigatus* to understand why *A. nidulans* infections are rarely observed and only seen in CGD.

Live-cell imaging was used to define the dynamics of recognition and phagocytosis by macrophages. Recognition and phagocytosis of *A. nidulans* compared to *A. fumigatus* was slower, which could be attributed to slower macrophage migration. Moreover, slower phagosome acidification was observed upon phagocytosis of *A. nidulans*. Similarly, a lower oxidative burst was observed following exposure to *A. nidulans*. In contrast, *A. nidulans* induced significantly higher concentrations of cytokines. Collectively, *A. nidulans* has a higher immunostimulatory and lower ROS-inducing capacity, yet is engulfed at a slower rate compared to *A. fumigatus*. Therefore, the slower phagocytosis caused by *A. nidulans* itself in addition to the specific defective phagocytic machinery that already makes CGD patients extremely susceptible to aspergillosis might explain why *A. nidulans* is specifically seen in this disease and not in other disease settings.

INTRODUCTION

Aspergillus species are environmental molds that play essential roles in carbon recycling of decaying organic debris. On a daily basis, humans are estimated to inhale hundreds of spores, yet these spores are efficiently removed from the lung resulting in the fact that healthy individuals only in very rare cases develop an *Aspergillus* infection.

However, certain patient groups have an elevated risk of developing aspergillosis; this susceptibility strongly depends on the status of the host immune system. Patients who are immunocompromised as a result of immunosuppressive therapy are highly susceptible to develop invasive aspergillosis (1). This mainly concerns patients with a secondary immunodeficiency due to chemotherapy or treatment with immunosuppressive drugs in the context of malignancies, organ or hematological stem cell transplantation (1-6). Such treatments are a major risk factor due to their suppression of the first line of antifungal host defence in the lungs (3-6). Although patients with primary immunodeficiency are not usually susceptible to aspergillosis, individuals with genetic dysfunction of the NADPH-oxidase complex, called chronic granulomatous disease (CGD), are highly susceptible to *Aspergillus* infections (7-10). Five genes are known, in which mutations can lead to the reduced activity of the NADPH-oxidase with a subsequent loss of the respiratory burst, mainly in neutrophils and macrophages resulting in a reduced killing capacity due to defective LC3 associated phagocytosis (11-13).

Interestingly, aspergillosis in CGD patients presents as less severe and is associated with a lower mortality of 25-27 % compared to 50-60% in hemato-oncological patients (7). The epidemiological distribution of the different *Aspergillus* species is also significantly different (14). *A. fumigatus* is the most commonly isolated species (62% SD±16.1) in invasive aspergillosis of hemato-oncological patients, followed by *A. flavus*, (17%, SD±17.5), *A. terreus* (10%, SD±10.1) and *A. niger* (2%, SD±2.5); infections with *A. nidulans* are much less common and account for 1% (SD±0.9) (14-20). Interestingly, no higher mortality has been attributed to the different species (16). In contrast, within the group of CGD patients that develop aspergillosis, infections caused by *A. fumigatus* are lower in number (48% SD±20), and a significantly higher number of infections are caused by *A. nidulans* (33% SD±12.7) (8, 9, 21, 22) (Supplemental figure 1A). Although *A. nidulans* is still less prevalent than *A. fumigatus* in CGD, its disease severity and mortality is significantly higher (9, 21, 22). Taken together, these studies point towards elevated mortality associated with *A. nidulans* infections (41% SD±15 vs 12% SD±13) (Supplemental figure 1B).

There is an interesting epidemiological difference when comparing the environmental prevalence of the different *Aspergillus* species. *A. fumigatus* is the most common airborne environmental fungus (1) and more prevalent compared to *A. nidulans*, 54% vs. 4% in a Spanish study (23). In contrast, in an Indian study *A. fumigatus* was as common

in the environment as *A. nidulans* (24). Although there are strong seasonal and regional differences, these studies suggest an overall higher prevalence of *A. fumigatus* in the environment compared to *A. nidulans* (25). Apart from the better air dispersibility of *A. fumigatus* conidia due to its higher hydrophobicity (26), the differences in prevalence of cases associated with both species suggest that factors such as differences in activation of the host response may determine the pathogenicity of *A. fumigatus* and *A. nidulans.* The high prevalence of *A. nidulans* in CGD patients cannot simply be attributed to the loss of ROS, since the *A. nidulans*-specific immune response has been shown to be independent of the NADPH-oxidase complex (27). To date, no studies have investigated and compared the initial immune recognition and phagocytosis of *A. nidulans versus A. fumigatus*.

By deciphering different aspects of the innate immune responses induced by *A. fumigatus* and *A. nidulans* under intact NADPH-oxidase conditions, we compared the immunostimulatory capacity of *A. nidulans versus. A. fumigatus.* Differences in the level of phagocytosis, cytokine induction and oxidative burst of phagocytes might help to explain why A. nidulans infection is only observed in CGD When a certain threshold of phagocytic clearing is reached *Aspergillus* will have the chance to keep growing and cause infection. Therefore, we focus on investigating phagocytosis dynamics of *A.fumigatus* and *A. nidulans* using live cell imaging to find a potential explanation for the higher incidence and mortality associated with *A. nidulans* in CGD patients.

EXPERIMENTAL PROCEDURES

Aspergillus species

Aspergillus conidia were cultured and harvested as described in a previous study (56). Resting conidia of *A. fumigatus* (CBS 101355/ATCC MYA-4609) and *A. nidulans* (CBS 114.63) were used at a final concentration of $1x10^7$/ml either live or heat-killed (30 minutes at 95˚C in a water-bath) for the PBMC stimulation assays. Live conidia were germinated and opsonized by incubation for 4 hours at 37˚C in 10% human serum and used for the ROS assay.

Live cell imaging

Murine J774.A1 macrophages were seeded in 8 well ibidi imaging dishes at a density of $1x10^5$/well and were allowed to adhere overnight. Live-cell video microscopy phagocytosis assays were carried out at 37˚C using an Ultra-VIEW VoX spinning disk microscope (Nikon, Surrey, United Kingdom). Volocity software was used for data analysis (version

6.3.1, Improvision, PerkinElmer, Coventry, United Kingdom). Immediately prior to live-cell imaging, DMEM was replaced with 200 mL prewarmed supplemented CO_2-independent medium (Gibco, Invitrogen, Paisley, United Kingdom). Live *A. fumigatus* or *A. nidulans* cell suspensions of resting conidia or 4 h swollen conidia were added to macrophages at a multiplicity of infection (MOI) of 1:1. In experiments with J774.A1 macrophages, acidic compartments were stained with 1 mM LysoTracker Red DND-99 (LTR) (Invitrogen). Volocity software (Improvision) was set to capture images every minute for a 6 h period using an electron-multiplying charge-coupled device (EMCCD) camera. For all conditions, at least two independent experiments were carried out, with a minimum of three movies per experiment.

Volunteers

Blood was collected from healthy volunteers by venous blood puncture after informed consent was obtained. All experiments were performed and conducted in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethical Committee (nr.2010/104).

PBMC isolation

Venous blood was drawn in 10 mL EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using *Ficoll*-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI+, consisting of RPMI-1640 culture medium (Dutch modification, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/mL gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the concentration was adjusted to $1x10^7$ cells/mL.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning, NY, USA) at a final concentration of $2.5x10^6$ /mL in an end volume of 200 µL per well. Stimulations were performed in the presence of 10% human serum. Cells were incubated at 37˚C with 5% CO_2, after stimulation either for 24 hours or 7 days supernatants were collected and stored at -20˚C.

Cytokine measurements

The innate cytokines IL-1β, IL-6, TNFα and IL-1Ra and the adaptive cytokines IFNγ, IL-5 and IL-17 were measured in the cell culture supernatants using commercial ELISA kits (IL-1β, TNFα, IL-1Ra, IL-5, IL-17: R&D Systems, Minneapolis, MN, USA; IL-6, IFNγ: Sanquin,

Amsterdam, The Netherlands) according to the instructions supplied by the manufacturer.

Killing of Aspergillus by human macrophages or PBMCs

Following differentiation human macrophages ($1x10^5$) or freshly isolated PBMCs ($5x10^5$) were exposed to *Aspergillus* conidia ($2x10^6$) in 96 well plates a final volume of 200µl. After 24 hours at 37ºC the cells were washed in water and plated in serial dilution on sabouroud agar plates. CFUs were counted after 24 hours at 37ºC.

ROS induction

The induction of reactive oxygen species were measured by oxidation luminol (5-amino-2,3,dihydro-1,4-phtalazinedione). PBMCs ($5x10^5$) were resuspended in HBSS and put in dark 96 well plates. Cells were exposed to HBSS, *A. fumigatus* heat inactivated resting conidia, live resting conidia, live germinated conidia (all at $1x10^7$/mL), *A. nidulans* heat inactivated resting conidia, live resting conidia, live germinated conidia (all at$1x10^7$/mL) or Zymosan (150 µg/mL) and immediately 20 µL of 1 mM luminol was added. Chemiluminescence was measured in BioTek Synergy HTreader at 37˚C for every minute during one hour.

Statistical analysis

Experimental data was plotted and analyzed using the GraphPad Prism V6.0 (GraphPad Software, Inc., USA). Results are shown as mean ± standard errors of the mean (SEM). The Mann-Whitney U-test and two-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests, were used to test statistical significance (* = $p<0.05$; ** = $p<0.01$;*** = $p<0.001$; and **** = $p<0.0001$).

RESULTS

Phagocytosis of *A. nidulans* and *A. fumigatus*

Live cell imaging was performed to investigate whether there are differences in recognition and subsequent phagocytosis of *A. nidulans* and *A. fumigatus*. Cells of the murine macrophage cell line J774.A1 were allowed to engulf freshly isolated *A. fumigatus* or *A. nidulans* spores for six hours. First, phagocytosis efficiency was assessed at the end of the 6 hours exposure to investigate whether there are major differences in overall phagocytosis of *A. fumigatus* and *A. nidulans*. Over the 6h time period J774.A1 macrophages were able to engulf freshly isolated *A. fumigatus* and *A. nidulans* spores with

equal efficiency. No significant difference was observed in the percentage of engulfed spores (figure 1A), or the number of spores that germinate at the end of the experiment (figure 1B). When looking at the macrophages that were exposed to *A. fumigatus* or *A. nidulans* at a MOI of 1:1, half of the macrophages engulfed spores and no significant differences were observed in the number of spores engulfed per macrophage (figure 1C) or the number of macrophages that undergo apoptosis (figure 1D).

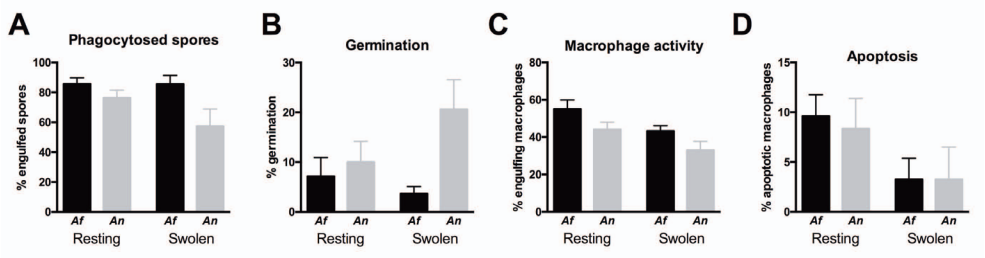

Figure 1: *Comparison of phagocytosis of A. fumigatus and A. nidulans by J774.A1 murine macrophages*

(A) Percentage of engulfed live resting and 4h swollen *A. fumigatus* (*Af*) and *A. nidulans* (*An*) spores after 6h of exposure to J774.A1 murine macrophages at a MOI of 1:1. (B) Percentage of germinating spores within the population of non-phagocytosed spores. (C) Macrophage activity assessed by the percentage of macrophages that engulfed spores (left panels) and within all macrophages an assessment of the number of spores engulfed per macrophage (right panels). (D) Percentage of macrophages that undergo apoptosis within the 6h experiment. Data is presented as the mean ±SEM and * = $p<0.05$.

Macrophages engulf *A. nidulans* at a slower rate compared to *A. fumigatus*

To dissect the dynamics of recognition and phagocytosis of *A. fumigatus* and *A. nidulans* in more detail, live cell imaging videos were analyzed to assess differences in recognition of fungal spores by macrophages (see video 1 for representative live cell imaging video). The moment of contact, defined as the moment of cell-cell contact between the macrophage and the spores, was assessed. A significant difference was observed in the moment of contact between cells and spores of *A. fumigatus versus A. nidulans* (figure 2A), which indicated slower recognition. Fifty percent of all *A. fumigatus* spores are recognized within 30 minutes, while this took over 1 hour for *A. nidulans* (figure 2A). Subsequently, the time of engulfment was assessed that was defined as the time between contact and full enclosure (figure 2B,C). However, no significant difference in the mean time of engulfment was observed (figure 3D) or the dynamics of engulfment (figure 2D). Therefore, we observed that dynamics of phagocytosis of *A. fumigatus* was different from *A. nidulans*, with *A. fumigatus* having significantly faster contact with phagocytic cells compared to *A. nidulans* spores (figure 2D).

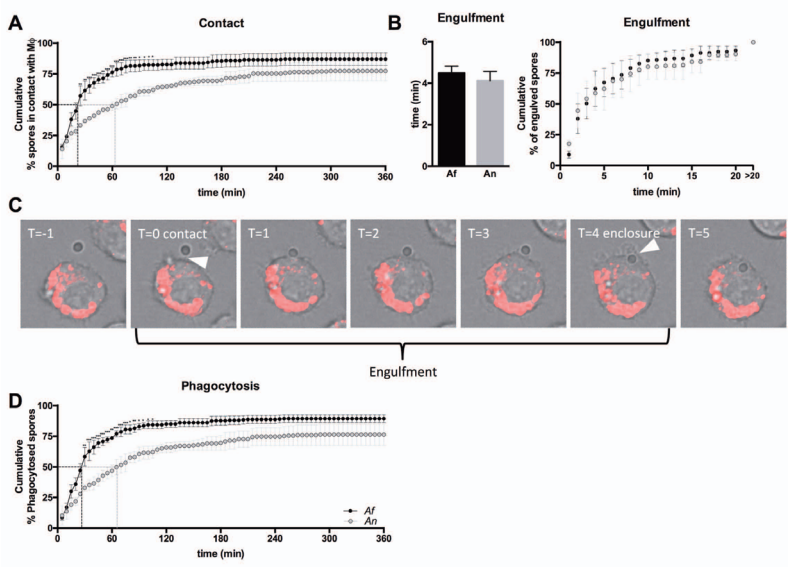

Figure 2: *Macrophages engulf A. nidulans at a slower rate compared to A. fumigatus*

(A) Cumulative percentage of *A. fumigatus* and *A. nidulans* spores in contact with J774.A1 macrophages. Contact is defined as the time-point where the macrophage makes visible physical contact with the spore. (B) Histogram of engulfment, defined as the time taken between contact and full enclosure of the fungus set out as percentage of events monitored in each minute. (C) Representative frame series illustrating the definition of contact and full enclosure. (D) Cumulative percentage of *A. fumigatus* and *A. nidulans* spores engulfed by J774.A1 macrophages.. All bars and dot plots represent mean ±SEM and * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001.

Macrophages migrate faster to engulf *A. fumigatus* spores than *A. nidulans* spores

To explain the decreased time to the engulfment of *A. nidulans* spores macrophage migration was analyzed during incubation of phagocytes with each fungal species. For individual engulfed spores we investigated whether macrophages actively migrated to engulf the spore. No significance difference was observed in the number of macrophages migrating towards resting spores. Although macrophages migrated significantly more towards swollen spores, no significant differences in the percentage of macrophages that migrated were observed between *A. fumigatus* and *A. nidulans* (figure 3A). In addition, no significant differences were observed in the distance that macrophages migrated to engulf spores. (figure 3C). However, macrophages migrated significantly faster towards *A. fumigatus* resting and swollen conidia than to *A. nidulans* (figure 3D).

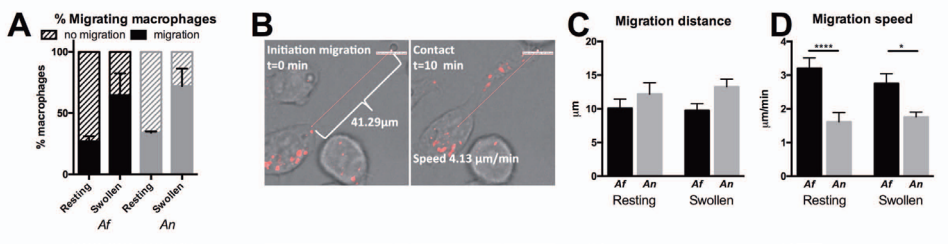

Figure 3: *A. fumigatus is more efficiently recognized and phagocytosed than A. nidulans*

(A) Percentage of macrophages that show active migration to *A. fumigatus* or *A. nidulans* resting spores or swollen spores. (B) Representative frames showing measurement of the distance of migration as well as the definition of contact between spore and macrophage. (C) Mean distance (mm) measured using velocity software from at the moment of initiation of macrophage migration. (D) Migration speed (mm/min) measured by velocity software from the moment of movement initiation to the moment of contact between macrophage and spore. All bars and dot plots represent mean ±SEM and * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001.

Phagosome acidification is slower for phagosomes containing *A. nidulans*

Phagosome acidification has previously been shown to be crucial for the killing of *A. fumigatus* (28). Lysotracker red (LTR) was used to stain the acidic compartments of macrophages. Following engulfment, the timing of phagosome acidification was assessed by localization of LTR as a halo around the engulfed spore (figure 4A). Phagosome acidification of *A. nidulans*-containing phagosomes was significantly slower than phagosome acidification of *A. fumigatus*-containing phagosomes (figure 4B). Analysis of the dynamics reveal that in the case of *A. fumigatus* 50% of engulfed spores are in acidified compartments under 8 minutes after engulfment while for *A. nidulans* this took over 11 minutes (figure 4C). This could indicate faster processing to phagolysosomes, yet additional markers are required to verify this.

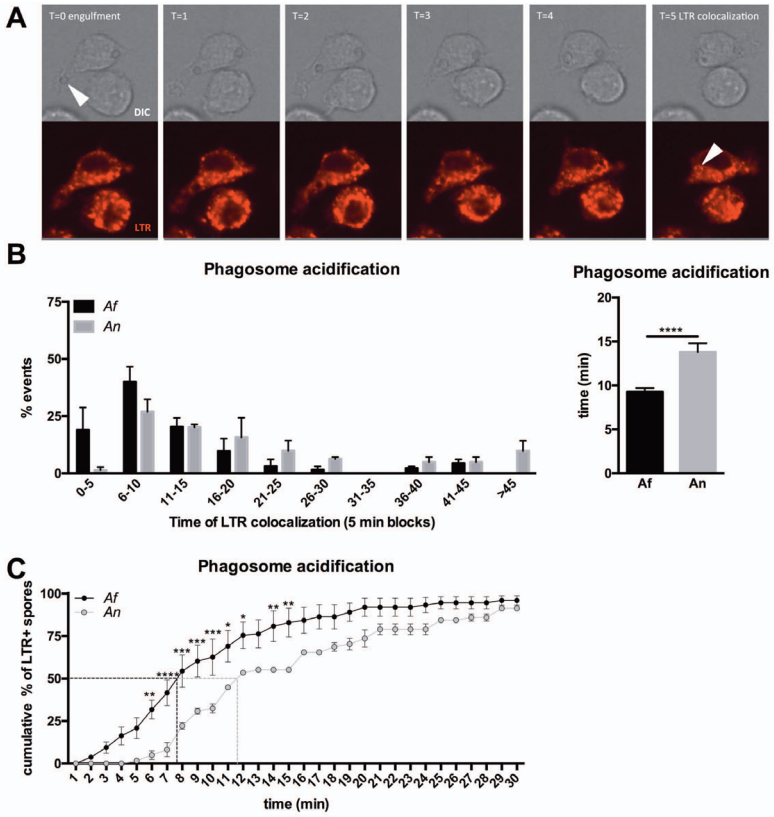

Figure 4: *A. nidulans containing phagosomes show delayed phagolysosomal fusion*

(A) Representative image series showing lysotracker red (LTR) staining of acidic compartments and appearance of a LTR-halo surrounding the conidia at 5 minutes post engulfment. (B) Histogram illustrating the distribution of the time after engulfment that LTR halo co-localized with the engulfed spore and a plot of the average time of LTR-halo co-localization compared between *A. fumigatus* and *A. nidulans* engulfed spores. (C) Cumulative dynamics of LTR halo co-localization. All bars and dot plots represent mean ±SEM and * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001.

A. fumigatus induces significantly stronger oxidative burst than *A. nidulans.*

The oxidative burst is thought to be essential for successful killing of *Aspergillus* conidia, since previously NADPH induced ROS was found to regulate LC3 associated phagocytosis (13, 29); a mechanism that is essential for fungal killing and antigen presentation (29-32) Therefore the capacity to induce an oxidative burst was compared between *A. fumigatus* and *A. nidulans* (Figure 5 A-D) In line with previously studies (27), we could confirm that *A. nidulans* shows a significantly impaired capacity to induce an oxidative burst in human PBMCs (Figure 5 C,D)).

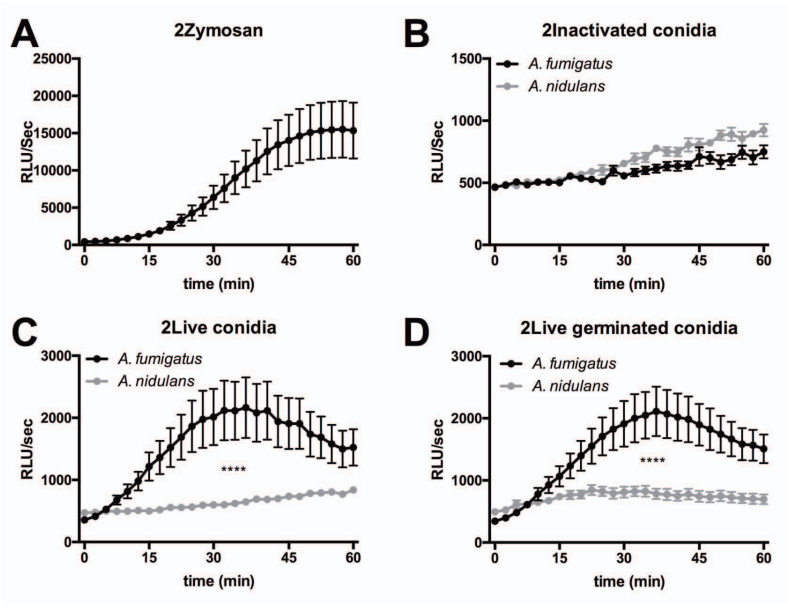

Figure 5: *A. fumigatus induces significantly higher ROS in human PBMCs compared to A. nidulans.*

(A-D) Human PBMCs (5x10^5 cells/well) of healthy volunteers (n=6) were pre-incubated with luminol before (A) Zymosan and RPMI, (B) heat-killed conidia of *A. fumigatus* and *A. nidulans* (1x10^7/ml), (C) live resting conidia of *A. fumigatus* and *A. nidulans* (1x10^7/ml), and (D) live germinated conidia of *A. fumigatus* and *A. nidulans* (1x10^7/ml were added. Total integrated ROS production is depicted for two independent experiments. All bars and dot plots represent mean ±SEM and * = p<0.05, an ** = p<0.01 Statistical analysis was performed with two way ANOVA for ROS assays and the Mann-Whitney-U test for cytokine data.

A. nidulans-induced cytokine levels are significantly higher compared to *A. fumigatus*

Previously a defective ROS production has been associated with a higher cytokine inducing capacity in CGD patients (33). Therefore, we investigated whether the reduced capacity of *A. nidulans* to induce an oxidative burst correlated with an increased cytokine response by stimulating human PBMCs of healthy volunteers with heat-killed and live conidia of *A. fumigatus* or *A. nidulans*. *A. nidulans*-induced cytokine production was indeed significantly higher for the innate cytokines IL-6, TNFα, IL-β and IL-1Ra after stimulation with both live and heat-killed conidia (Figure 6A-D). Only the induction of IL-1Ra and TNFα by live conidia did not significantly differ, although the same trend towards a higher immune induction could be observed.

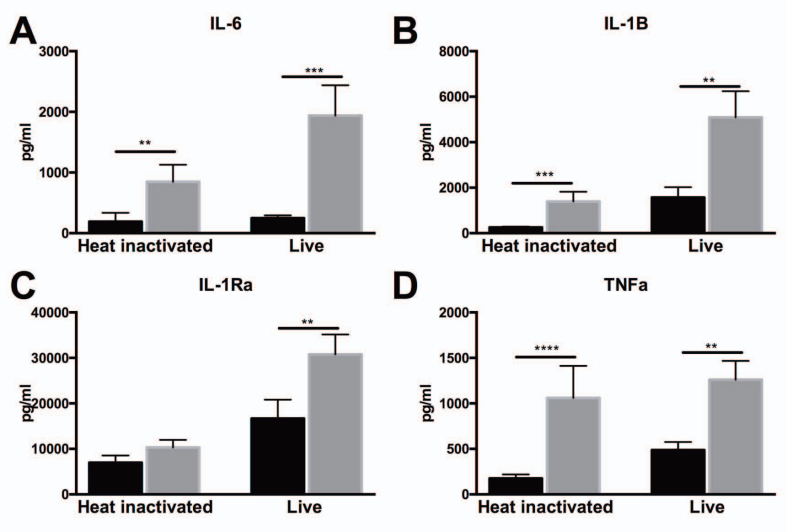

Figure 6: *A. fumigatus induces significantly lower innate cytokines in human PBMCs compared to A. nidulans.*

(A-D) PBMCs of healthy volunteers (n=7-16) were stimulated with live or heat-killed conidia of *A. fumigatus* and *A. nidulans*. The innate cytokines (A) IL-6, (B) IL-1β, (C) TNFα or (D) IL-1Ra were measured in the cell-culture supernatant by ELISA. All bars and dot plots represent mean ±SEM and * = p<0.05, an ** = p<0.01 Statistical analysis was performed with two way ANOVA for ROS assays and the Mann-Whitney-U test for cytokine data.

DISCUSSION

In this study, various aspects of the innate immune response against *A. fumigatus* and *A. nidulans* were compared, to identify differences in phagocytic and immune-stimulatory capacities that might further explain their differential pathogenicity in certain patient settings such as CGD. Although macrophage phagocytosis and activation were comparable at a fixed end point of 6 hours, both species differed markedly in their engulfment and uptake kinetics during interactions with host cells: *A. fumigatus* was recognized and taken up much faster compared to *A. nidulans*. Strikingly, phagosomes containing *A. fumigatus* were significantly faster acidified compared to spores of *A. nidulans*. This could indicate faster processing to phagolysosomes, yet additional markers are required to verify this. Although *A. nidulans* spores are less efficiently phagocytosed, human PBMCs exposed to this species responded in a stronger pro-inflammatory manner, reflected by the high cytokine induction, yet its capacity to induce an oxidative burst was significantly lower than for *A. fumigatus* (Figure 7).

This is one of the first studies to investigate the dynamics of *Aspergillus* phagocytosis. Using live cell imaging to compare the phagocytic dynamics of the two species *A.*

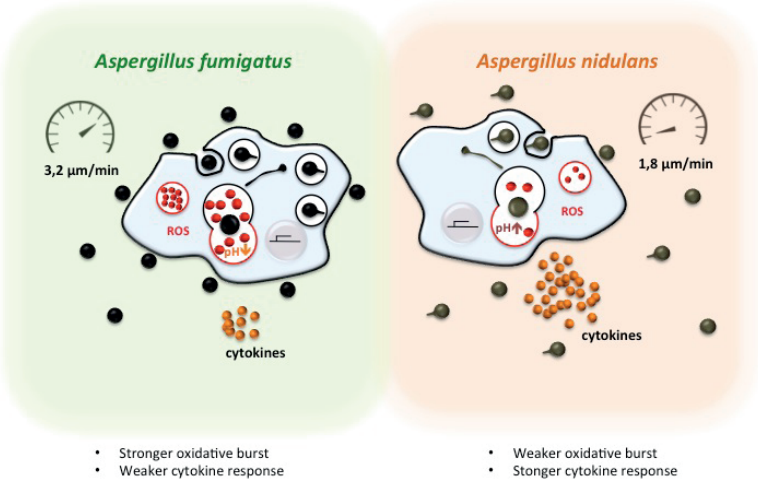

Figure 7: *Schematic comparison of the innate immune response against A. fumigatus and A. nidulans*

The innate immune response against *A. nidulans* differs from the immune response against *A. fumigatus* in delayed recognition, delayed phagocytosis, slower macrophage migration, slower phagosome acidification, less pronounced oxidative burst and a higher levels of cytokine production.

nidulans and *A. fumigatus* lead to some general observations that could be of broader interest. We observed that macrophages migrated poorly to resting spores of either *A. fumigatus* or *A. nidulans*, while following germination of the spores macrophages migrated more actively towards the fungi. This is different from other fungi, for example live cell imaging with *Candida albicans* revealed active migration towards the fungi irrespective of whether they are as yeast or hyphae (34). A potential explanation for the slower phagocytosis of resting *Aspergillus* spores might be the hydrophobin sheet that masks molecules on the surface of the cell wall (35, 36), and upon germination (when the hydrophobin layer is removed) immune-stimulatory molecules on the cell wall are recognized. An interesting aspect is that macrophages sense the germination over a distance (video 2), suggesting the macrophages might sense a gradient of secreted fungal molecules. Alternatively, macrophages may have superfine projections that are in contact with the spores and sense its changing cell wall, as was previously revealed for *Candida* by scanning electron microscopy (37). Nevertheless, the factors that trigger the macrophages to migrate towards germinating spores warrants further investigation. For *C. albicans* it also has been observed that the fungi form hyphae inside the macrophage, thereby lysing and escaping from macrophages (38). This phenomenon was not observed during phagocytosis of *A. fumigatus* and *A. nidulans*, however when intracellular germination

occurred the hyphae were expulsed from the cell (video 3) as was previously described for *Candida* and *Cryptococcus* (39-41). These observations provide new insights into the interplay between macrophages and *Aspergillus*, and highlight the potential of live cell imaging as a tool to study dynamics of phagocytosis.

A crucial goal of this study was to understand why the susceptibility to *A. nidulans* in CGD patients is much higher compared to hemato-oncological patients, despite the latter having more profound immunosuppression. When discussing this point, the environmental distribution and exposure of both patients groups must also be taken into account (Figure S1). Although fundamental studies are lacking, it can be assumed that *A. fumigatus* is the more abundant pathogen in the environment (23, 42). While hemato–oncological patients are more severely immunocompromised with sometimes a complete abolishment of white blood cells but a normal killing activity of residual cells, such as residential alveolarmacrophages, CGD patients have normal cell counts with a defect in fungal killing by neutrophils, macrophages and monocytes. Although the primary defect in CGD patients is an inability to produce NADPH dependent ROS, killing of *A. nidulans* is independent of ROS induction (27). However, the defect in the NADPH complex in CGD patients has also been associated with a defect in LC3 associated phagocytosis (29, 43), a form of phagocytosis that requires the autophagy machinery for more efficient processing of engulfed pathogens for killing (29-31) and MHCII mediated antigen presentation (44). In addition, neutrophils of CGD patients have defective neutrophil extracellular trap (NET) formation. Restoration of either LC3 associated phagocytosis (13) or NET formation by gene therapy (45) in CGD mice/patient was successful for controlling aspergillosis respectively. We demonstrated that in addition to the defects in a CGD cell, *A. nidulans* phagocytosis is already delayed in a healthy phagocytic cell. In addition to the fungal clearance defects in cells of CGD patients (that account for both *A. fumigatus* and *A. nidulans*), there is a delayed phagocytosis and processing of *A. nidulans* spores. This might help to explain the incidence of *A. nidulans* in this disease, but also the higher mortality associated with *A. nidulans* infections in CGD (9, 21, 22). Hemato–oncological patients experience a relatively short period of time at risk for *Aspergillus* infections, while CGD patients are exposed to environmental conidia over years. Therefore, the net result of an already defective *Aspergillus* phagocytic machinery characteristic for CGD plus the slower phagocytic rate of *A. nidulans* might explain why *A. nidulans* infections are only seen in CGD and not in other diseases setting where this severe threshold of defective phagocytosis for *A. nidulans* is not reached. In severely immunocompromised patients the epidemiological distribution of infections follows the burden of spores in the environment, which seems to be the result of the chance of inhalation of environmental

spores. In contrast, in CGD patients the delayed and less efficient engulfment of *A. nidulans* might be more important for explaining their increased susceptibility to *A. nidulans*.

The higher immunostimulatory capacity of *A. nidulans* compared to *A. fumigatus* is in line with earlier studies. These studies observed a higher cytokine stimulatory capacity with *A. nidulans* compared to *A. fumigatus* using different strains than in this study (33). Interestingly, CGD patients, in which *A. nidulans* is much more prevalent compared to hemato-oncological patients, demonstrated a pro-inflammatory phenotype (46-49). This hyper inflammation in CGD patients with high interleukin-1 responses (13, 33) has been associated with colitis (13). On top of the hyper inflammation in CGD patients, we observe that *A. nidulans* induces much more potent cytokine responses than *A. fumigatus*. During an infection the higher immunostimulatory capacity of *A. nidulans* could lead to even higher pro-inflammatory immune responses. Failure to control excessive inflammatory responses has been associated with a poor clinical outcome and increased mortality from aspergillosis (50, 51). It is therefore tempting to suggest that the higher mortality observed with *A. nidulans* could also be as a result of excessive inflammatory reactions leading to immunopathology.

It remains to be determined why *A. nidulans* is more capable of inducing pro-inflammatory cytokines, although differences in cell wall architecture between *A. nidulans* and *A. fumigatus* certainly exist. Galactosaminogalactan (GAG) is an important anti-inflammatory polysaccharide of the cell wall of *A. fumigatus* that induces the anti-inflammatory cytokine IL-1Ra (52). The absence of GAG in the cell wall of *A. nidulans* (53) could potentially explain its higher capacity to stimulate pro-inflammatory cytokines.

Furthermore, a high immunostimulatory capacity associated with high levels of the pro-inflammatory IL-1β or TNFα can be the result of continuous extracellular signaling via pattern recognition receptors and activation of the subsequent signaling pathways. Frustrated phagocytosis of long filaments is a known phenomenon associated with the production of high levels of pro-inflammatory cytokines (54, 55). Although these filaments were much bigger than conidia, we observed differential phagocytosis of the fungi, and a continuous presence of extracellular filaments might drive more pro-inflammatory responses.

Collectively, our data provides novel insights in the dynamics of *Aspergillus* phagocytosis and illustrates clear differences between *Aspergillus* species, with, *A. nidulans* spores being much less efficiently engulfed by macrophages. Alternatively, *A. nidulans* shows a higher capacity to stimulate cytokine responses. These findings might contribute to explain why *A. nidulans* infections are specifically observed in CGD and are associated with a higher mortality.

Video 1:
Representative video of J774.A1 macrophages engulfing *A. fumigatus* spores at 1000x magnification captured at 1 image per minute and played back at 12 frames per second.
Video 2:
Representative video of J774.A1 macrophages targeting germinating *A. fumigatus* spores at 1000x magnification captured at 1 image per minute and played back at 12 frames per second.
Video 3:
Representative video of J774.A1 macrophages engulfing *A. fumigatus* spores and expulsing them following intracellular germination. Captured at 1000x magnification captured at 1 image per minute and played back at 12 frames per second.

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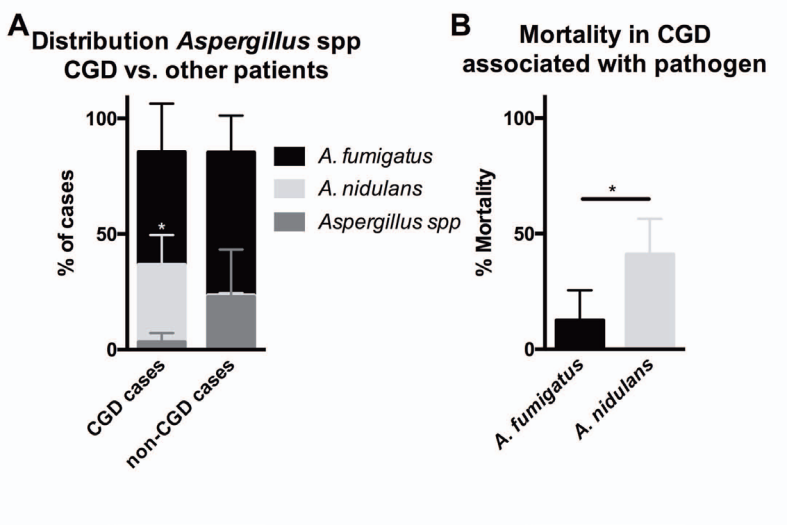

Figure S1: *Differences in causative pathogens for aspergillosis between CGD and other patients*

(A) Distribution of *Aspergillus* species in aspergillosis cases in CGD patients *versus* cases in non-CGD patients based on literature search (8, 9, 14-22). (B) Mortality rate in CGD patients due to the fungal infection stratified on causative pathogen. The bars represent mean percentage ±SEM and * = $p<0.05$. Statistical analysis was performed with an unpaired t test with Welch's correction.

CHAPTER 8

Th17 cytokines deficiency in patients with *Aspergillus* skull base osteomyelitis

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ABSTRACT:

Background: Fungal skull base osteomyelitis (SBO) is a severe complication of otitis externa or sinonasal infection, and is mainly caused by *Aspergillus* species. Here we investigate innate and adaptive immune responses in patients with *Aspergillus* SBO to identify defects in the immune response that could explain the susceptibility to this devastating disease.

Methods: Peripheral blood mononuclear cells isolated from six patients with *Aspergillus* SBO and healthy volunteers were stimulated with various microbial stimuli, among which also the fungal pathogens *Candida albicans* and *Aspergillus fumigatus.* The proinflammatory cytokines IL-6, TNFα and IL-1β, and the T-helper cell-derived cytokines IFNγ, IL-17 and IL-22 were measured in cell culture supernatants by ELISA.

Results: Proinflammatory cytokine responses did not differ between SBO patients and healthy volunteers. The *Candida*- and *Aspergillus*-specific Th17 response (production of IL-17 and IL-22) was significantly decreased in the SBO patients compared to healthy individuals, while Th1 cytokine response (IFNγ production) did not differ between the two groups.

Conclusions: We show that patients with *Aspergillus* skull base osteomyelitis infection have specific defects in Th17 responses. Since IL-17 and IL-22 are important for stimulating antifungal host defense, we hypothesize that strategies that have the ability to improve IL-17 and IL-22 production may be useful as adjuvant immunotherapy in patients with *Aspergillus* SBO.

BACKGROUND

Skull base osteomyelitis (SBO) is a rare but life-threatening infection, which originates either from the external ear canal or the paranasal sinus. Infiltrative growth from the external acoustic duct into the temporal bone is also termed malignant or invasive otitis externa [1]. This aggressive infection is usually caused by *Pseudomonas aeruginosa*, sometimes by fungal pathogens (mainly *Aspergillus* spp.), and in a minority of cases by other bacteria (such as S*taphylococci*, *Proteus* and *Klebsiella*) [2-4]. Besides an otologic origin of infection, sinusitis is the second major cause of skull base osteomyelitis. Although this is a rare complication, infections of the frontal, ethmoid, sphenoid and maxillary sinus can spread to the orbital and frontal bone, clivus and petrous apices. A distinct form of sinusitis is fungal rhinosinusitis. This has a broad clinical spectrum ranging from chronic forms with gradually progressing osteomyelitis to necrotizing angioinvasive disease. *Aspergillus, Rhizopus* and *Fusarium* are the most commonly identified fungi. Fungal skull base osteomyelitis due to *Aspergillus* is an infection with considerable morbidity and mortality rates up to 50%. In addition to aggressive surgical debridement and systemic antifungal therapy, the mainstay of therapy includes, whenever possible, correction of the underlying immunologic defect.

Spores of *Aspergillus* are continuously inhaled and therefore fungal colonization of the upper airways is common. Despite this continuous exposure, invasive disease caused by *Aspergillus* in an immunocompetent host is very rare. Although some degree of immunosuppression may be present in patients with fungal SBO, often the only apparent risk factor identified is a chronic external otitis or an anatomical obstruction of the sinuses (e.g. nasal polyps or chronic inflammation of the mucosa). The extent of tissue invasion in these patients may vary depending on the underlying immune status of the host. In the present study we present six cases of skull base osteomyelitis due to *Aspergillus fumigatus* and *Aspergillus flavus*, in whom we investigated whether the innate and adaptive immune responses known to be important for antifungal host defense are defective.

METHODS

Volunteers and Patients

We describe six patients who were admitted to the Radboud University Medical Center with a culture-proven invasive *Aspergillus* osteomyelitis of the skull. All patients were diagnosed between September 2007 and July 2010. Charts were reviewed for data on demographics, risk factors, presenting symptoms, treatment, side effects, microbiology results, and clinical outcome. Response was defined according to the revised MSG/

EORTC consensus group definition [5]. Patients and healthy volunteers, who served as healthy controls in the immunological experiments, were asked for blood donations. The blood samples were collected from patients and healthy volunteers after informed consent was obtained in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethics Committee (nr.2010/104).

Stimuli

E.coli lipopolysaccharide (LPS) (10 ng/ml) (TLR4 ligand, *E. coli* serotype O55:B5, Sigma-Aldrich St. Louis, MO USA); heat-killed *Staphylococcus aureus* clinical isolate (*S. aureus*) (1x107 /ml); heat-killed *Candida albicans* yeast ATCC MYA-3573 (UC820) (*C. albicans*) (1x105 /ml); heat-killed *Aspergillus fumigatus* clinical isolate V05-27 (*A. fumigatus*) conidia (1x107 /ml) were cultured and isolated as described previously [6].

Peripheral blood mononuclear cells (PBMCs) isolation

Fresh venous blood was drawn in 10 ml EDTA tubes from patients and controls and processed in parallel continuously from PBMC isolation to cytokine measurement. Every patient gave blood only once. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE Healthcare, Zeist, The Netherlands) density gradient centrifugation. The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in RPMI-1640 culture medium (Dutch modification, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10 µg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate (Gibco). The cells were counted with a particle counter (Beckmann Coulter, Woerden, The Netherlands) and the concentration was adjusted to 5x106 cells/ml.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning, NY, USA) at a final concentration of 2.5x106/ml in an end volume of 200 µl per well. Either medium or stimuli were added. Cells were incubated at 37˚C with 5% CO2, after 24 or 48 hours or 7 days supernatants were collected and stored at -20˚C. Seven-day stimulations were performed in the presence of 10% pooled human serum. All stimulations assays were performed in duplicates.

Cytokines measurements

Cytokines were measured in the cell culture supernatants using a commercial ELISA kit (IL-1β, TNFα, IL-17 and IL-22: R&D Systems; IL-6 and IFNγ: Sanquin) according to the instructions supplied by the manufacturer. Proinflammatory cytokines production was

measured after 24 hours, IFNγ after 48 hours and the T helper cytokines IL-22 and IL-17 after 7 days of incubation.

Statistical analysis

The Mann-Whitney-U test was used to detect differences between healthy controls and patients. A *p*-value of < 0.05 was considered statistically significant (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$). Graphs represent cumulative results of all performed experiments and are presented as mean ± standard error of the mean. Data were analyzed with GraphPad Prism v 5.0.

RESULTS

Demographic characteristics of patients and controls

Patients: Three male and three female patients were included in the study with age ranging from 37 to 87 years (mean: 59.5 years). Infections were located in the sphenoid, mastoid or ethmoid bones with partial affection of sinus cavernosus, frontal or temporal lobe or orbita. All patients presented with cranial nerve palsy. In four cases the diagnosis was additionally confirmed by a positive histology. One patient developed *Aspergillus* osteomyelitis following trans-sphenoidal surgery for pituitary adenoma with a chronic myeloid leukemia (CML) in the past; two had a history of diabetes; all other patients had primary fungal infection of sinus or mastoid.

Controls: Three male and three female healthy volunteers were included in the control group; the age ranged from 24 to 60 years (mean: 42.7 years). Three of the patients were recruited from the blood bank via the blood donor service (Sanquin, Nijmegen, The Netherlands) and three were recruited directly to our department for blood donation. All volunteers were healthy and did not have an immunologically relevant medical history.

Microbiology

Culture and molecular identification confirmed the fungal infection and susceptibility to antifungal drugs was tested (Table 1). Galactomannan assay was performed in all patients on serum and in 4 patients on cerebrospinal fluid. All results were negative (index < 0.5).

Table 1: *Anti-fungal susceptibility of microbiological isolates.*

MIC values of fungal isolates tested for Amphotericin B (AMT), Itraconazole (ITC), Voriconazole (VOR), Anidulafungin (Anidula), Posconazole (POSA) and Caspofungin (CASPO) are listed.

Case	Isolate	AMT (mg/L)	ITC (mg/L)	VOR (mg/L)	Anidula (mg/L)	POSA (mg/L)	CASPO (mg/L)
1	*A. fumigatus*	*					
2	*A. fumigatus*	1	1	2	0.063	0.25	
3	*A. fumigatus*	0.5	0.063	0.125		< 0.016	0.5
4	*A. fumigatus*	1	0.25	1	0.031	0.063	
5	*A. fumigatus*	**					
6	*A. flavus*	1	0.063	1		0.031	0.5

*MIC impossible because of poor sporulation. Analysis of Cyp51A-gene: no TR/L98H

**No isolate available for susceptibility testing

Treatment, clinical outcome and blood sampling

Surgical debulking was performed in all patients. All patients were initially treated with systemic antifungal drug therapy. Voriconazole was first line treatment in all patients. Four patients were treated with voriconazole monotherapy. One patient was concomitantly treated with liposomal amphotericine B during the first months of treatment. Posaconazole was used after induction treatment in two patients. Duration of therapy (from 4.5 to 35 months) was guided by clinical response and imaging, with follow-up from 8 to 38 months. Four patients had a complete response, one had a relapse, and one died due to respiratory failure. During infection the leukocytes count was normal in five of six patients, ranging from 6.6 x 109/l to 9.1 x 109/l. One patient had a decreased leukocyte count of 3.1 x 109/l and slightly decreased numbers in the differential blood count. Three patients had normal and three patients had increased CRP values (11 mg/l, 14 mg/l and 32 mg/l). Blood samples for immunological assays were taken after clinical improvement and response to the therapy: in three patients during the first month after onset of the antifungal therapy, while in three patients blood was collected after the pharmacological therapy had been finished and the infection did not recur.

Patients with *Aspergillus* SBO do not differ from healthy controls in their production of proinflammatory cytokines

To investigate the innate immune response, PBMCs isolated from six patients with *Aspergillus* SBO were stimulated with *Aspergillus fumigatus, E. coli* LPS and different pathogens and compared with healthy controls (Figure 1 A-C). Neither the unspecific LPS-stimulated, nor the pathogen-specific *C. albicans, S. aureus,* and disease-specific

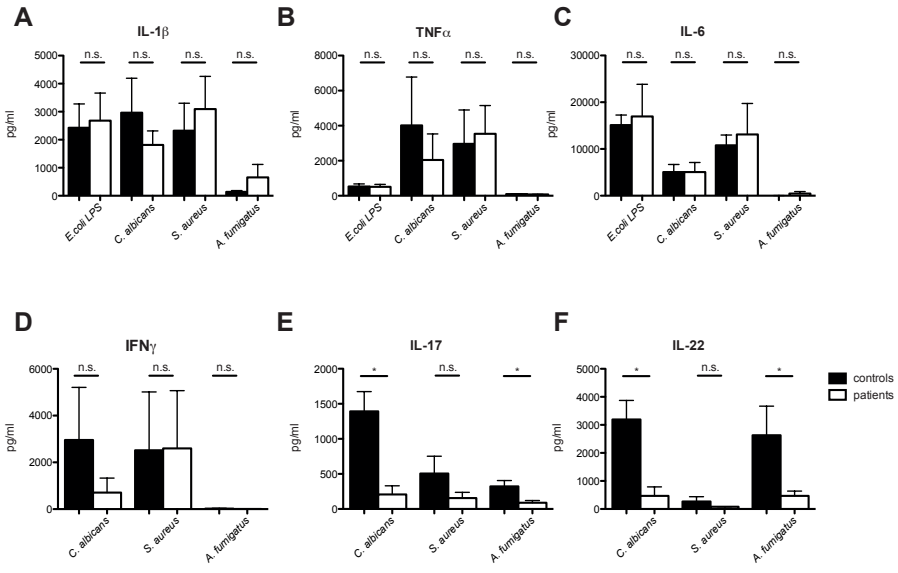

Figure 1: *SBO patients have an intact innate immune response, but are defective in IL-17 and IL-22 production.*

PBMCs stimulated with *E. coli* LPS, heat killed *C. albicans* yeast, *S. aureus* and *A. fumigatus* were cultured for 24 hours 48 hours or 7 days respectively. The innate cytokines IL-1β (**A**), TNFα (**B**), IL-6 (**C**) (after 24 hours) and adaptive cytokines IFNγ (**D**) (after 48 hours) and IL-17 (**E**) /IL-22 (**F**) (after 7 days) were measured in the cell culture supernatant by ELISA. Controls (black bars, n=6) compared with SBO patients (white bars, n=6).

A. fumigatus stimulations showed differences in the production of the inflammatory cytokines IL-1β, TNFα and IL-6.

Patients with *Aspergillus* SBO are deficient in *Aspergillus*-induced IL-17 and IL-22 but not in IFNγ production

While the recognition and initiation of inflammatory cytokine responses revealed to be intact, we wanted to address the question whether defects in the acquired immune response might explain the high susceptibility to the fungal infection of the six fungal SBO patients included in this study. PBMCs were stimulated with *C. albicans* and *S. aureus*, serving as positive controls for the induction of IFNγ, IL-17 and IL-22, and *A. fumigatus*, to investigate pathogen-specific deficiencies. The Th1 response, shown by IFNγ production, did not differ between patients with *Aspergillus* SBO and healthy controls (Figure 1D). In contrast, IL-22 and IL-17 production was significantly reduced after stimulation with both fungal pathogens *C. albicans* and *A. fumigatus* (Figure 1E-F).

DISCUSSION

In this study, we describe the clinical presentation and immunological features of six patients with *Aspergillus* SBO. None of the patients were neutropenic at the time of the infection or had a known primary immunodeficiency. We hypothesized that specific defects in *Aspergillus*-specific innate and/or adaptive immune response would contribute to the unsuccessful fungal clearance and extent of the *Aspergillus* infection in our patients. Therefore, we investigated host responses in six patients with *Aspergillus*-SBO. While the innate responses were not different from a healthy control group, Th17 cytokines induced by fungal pathogens such as *C. albicans* and *A. fumigatus* were shown to be defective in the patients with *Aspergillus* SBO.

Aspergillus spp. can cause several forms of diseases dependent on the site of infection and immune status of the host. *Aspergillus* osteomyelitis is increasingly being reported [7] with approximately 15% of the cases affecting the skull base [7, 8]. *Aspergillus* SBO is a severe complication of otitis externa or invasive sinonasal aspergillosis, in which most patients become infected via the tympanic cavity or the sinus [2].

The main risk factors described for invasive *Aspergillus*-SBO are systemic immunosuppression [7] and hematologic malignancies (12%) [7, 9]. One patient included in this study suffered from chronic myeloid leukemia, and he was treated with the tyrosine kinase inhibitor imatinib at the time of the infection. Two patients had diabetes mellitus and three suffered from sinusitis, which have been reported in previous studies as risk factors [7, 9]. Although almost all patients had normal leukocyte counts (one was slightly decreased), additional factors influencing the immune response such as the imatinib treatment, diabetes or the high age of one patient might affect the IL-17 response as well. Normal monocyte-derived cytokine levels point to a specific T-cell defect.

Early recognition and therapeutic intervention in invasive sinonasal aspergillosis with systemic antifungal therapy and surgical resection and/or debridement is important. In accordance with the current guidelines, all patients included in the present study were treated with surgical debridement of the infected bone and systemic antifungal drug therapy, of which voriconazole was the drug of first choice. We observed a mortality of 1 out of 6 in the patients included in the present study, which is in line with the reported poor clinical outcome of *Aspergillus* osteomyelitis, which has a 25% 12-weeks mortality [10].

Why did our patients without an apparent severe immunodeficiency get invasive aspergillosis? *Aspergillus* spp. are an occasional commensal of the external ear and paranasal sinuses [11], but invasive disease is very rare. Chronic infection leading to damage of the epithelial barrier is an important entry for the fungus to infect the host. Recognition of *Aspergillus* will result in the production of proinflammatory cytokines that

will recruit immune cells to clear the infection [12]. In the present study, patients with *Aspergillus* SBO showed normal production of the cytokines TNFα, IL-1β and IL-6 after stimulation with *A. fumigatus, C. albicans*, the Gram-positive bacterium *S. aureus*, and the Gram-negative cell wall component *E. coli* LPS. Therefore, a defect in the production of monocyte-derived proinflammatory cytokines is unlikely to be the cause of the susceptibility of fungal SBO in our patients. Acquired adaptive T-helper responses also play an important role in anti-*Aspergillus* host defense. The protective role of IFNγ in the *Aspergillus*-specific host response has been reported previously [13]. We did not observe a difference in *Aspergillus*-specific IFNγ production; *Candida*-specific IFNγ production showed a trend towards a lower IFNγ production, but this was not significant. However, the *Aspergillus*-specific Th17 response was significantly lower in SBO patients compared to the healthy control group. In addition, this was also observed, when the cells were stimulated with *Candida.* IL-17 is a characteristic cytokine produced by Th17 cells. Th17 cells are crucial for neutrophil recruitment and controlling fungal invasion at the level of mucosae and skin. [14]. Similar to IL-17, the IL-22 production was significantly decreased in SBO patients compared to healthy controls in the present study. IL-22 is also produced by Th17 cells [15] and shares many effector functions with IL-17 [16]. IL-22 plays a predominant role in mucosal host defense [17] by inducing anti-microbial peptides produced by epithelial cells, which can kill microorganisms directly [18].

In a previous study we have identified that IFNγ treatment had beneficial effects on the immune status including IL-17 and IL-22 responses in a case series of patients with invasive fungal infections [19]. This suggests that adjuvant therapy with recombinant IFNγ may improve the outcome of patients with a severe fungal SBO. Another treatment option would be to increase the differentiation into Th1 and Th17 cells by GM-CSF [20]. GM-CSF was shown to enhance the secretion of inflammatory cytokines [21] and antigen-presentation [22] under inflammatory conditions. It also induces the differentiation of progenitors cells into monocytes and granulocytes [23]. Therefore, GM-CSF might have beneficial effects on immune cells in the skin even in the setting of an IL-17/IL-22 deficiency [24]. One might speculate about supplementary therapy with recombinant IL-22, since IL-22 was shown to have beneficial effect in wound healing processes and pre-clinical studies have shown good toleration of administration of the drug [25].

We are aware of the fact that the sample size of six patients is inevitably small. A type I error of 5%, meaning the likelihood to accept the hypothesis that *Aspergillus* SBO patients and control do not differ in their *Aspergillus*-specific IL-17 response was assumed. Using the calculated means, standard deviations of the experimental measurements and sample size of both groups we calculated a statistical power of 81%. Thus, the Type II error, meaning the hypothesis that patients and controls differ in their IL-17 response

is neglected, lay with 19% in an acceptable range for medical tests. Retrospectively, due to the differences observed, the sample size of 6 donors was the minimal size needed to detect statistically relevant differences. Further sample size independent calculation revealed an effect size of 1.5, which indicates that *Aspergillus* SBO has a statistically strong effect on the *Aspergillus*-specific IL-17 production.

Nevertheless, there are also some limitations of the study. One note of caution is that while patients and controls matched regarding their gender, the patients were in average older, although the 15 years difference is unlikely to explain the significant differences observed. Further, it remains unknown whether the defective IL-17 and IL-22 production was the consequence of a primary defect (e.g. genetic) or was secondary to a predisposing factor of the *Aspergillus* SBO patients (e.g. the antifungal treatment). Nevertheless, the present study contributes to understanding the specific defective host defense mechanisms underlying SBO due to *Aspergillus*.

This is the first study describing a deficiency in fungal-induced Th17 responses in patients with *Aspergillus* skull base osteomyelitis. Future studies are needed to validate this observation and its clinical implication, especially the potential beneficial effects of immunotherapy aimed to boost Th17 responses.

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20 mg/ml
mg = 1 ml

CHAPTER 9

Th2 and Th9 responses in patients with Chronic Mucocutaneous Candidiasis and Hyper IgE syndrome

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ABSTRACT:

Background: STAT1 mutations cause Chronic Mucocutaneous Candidiasis (CMC), while STAT3 mutations cause Hyper IgE syndrome (HIES). CMC and HIES patients have T helper(Th)17 defects suffering from mucosal Candida infections, but only HIES patients show an allergic phenotype with eczema, eosinophilia and high IgE levels.

Objective: We investigated whether differential Th2 and Th9 responses may explain the clinical differences.

Methods: Peripheral blood mononuclear cells of CMC patients (n=4), HIES patients (n=4), atopic dermatitis patients (n=4) and healthy volunteers (n=13) were stimulated with Candida and Staphylococcus aureus, with and without IL-4. The cytokines IL-5, IL-13, IL-9, IL-17 and TGFβ and regulatory T cells were measured in cell culture supernatants by ELISA or flowcytometry, respectively.

Results: PBMCs of CMC patients showed a significantly impaired production of the Th2 cytokines IL-5 and IL-13, especially in the presence of IL-4. Moreover, IL-9 production was significantly lower in CMC patients compared to healthy controls. In contrast, HIES patients and AD patients showed normal IL-5 and IL-13 production, while IL-9 production was significantly lower in HIES patients compared to healthy controls. Although TGFβ was involved in the IL-4-induced IL-9 production, TGFβ levels and the frequency of regulatory T cells did not differ between HIES patients and controls. Flowcytometry analysis demonstrated an $IL\text{-}9^{+}IL\text{-}17^{+}CD4^{+}$ subset in healthy controls after stimulation with Candida which was less present in HIES patients.

Conclusion: CMC patients have a general Th defect including Th2 and Th9, while HIES patients have normal Th2 cytokines. These differences are in line with their clinical presentation. Surprisingly, the allergic cytokine IL-9 was deficient in both HIES and CMC, suggesting a Th-17 derived origin.

INTRODUCTION

Atopic diseases such as asthma or atopic dermatitis are classically driven by an elevated T helper (Th) 2 response, characterized by the cytokines Interleukin (IL) -4, IL-5 and IL-13. These cytokines mediate a type 1 immediate hypersensitivity reaction associated with IgE class switch and eosinophil recruitment that is followed by a late phase allergic reaction caused by infiltrating allergen-specific T cells that lead to persistent allergic symptoms (1, 2). Next to the Th2 cells, other T helper cell subpopulations have been identified as important players driving pathological atopic reactions. This includes not only an imbalance between Th1 and Th2 cells (3) or between Th2 cells and regulatory T cell (4), but also disturbed immune responses of the more recently identified Th17 and Th9 cells. IL-17A (hereafter named IL-17), produced by Th17 cells, is an important pro-inflammatory cytokine in the host defence against extracellular pathogens, especially against *Candida* (5), but can also drive pathology in allergic airway inflammation, where IL-17 was identified to induce IgE-mediated late-phase asthmatic response (6) and granulocyte influx (7). IL-9, another pro-inflammatory cytokine derived from Th9 cells after differentiation from a naïve T cell in the presence of IL-4 and transforming growth factor (TGF) β, leads to mast cell activation, eosinophil recruitment and IgE class switch during allergic inflammation (8, 9). Neutralization of IL-9 in an ovalbumin-induced asthma model was shown to significantly reduce allergic symptoms (10) . Interestingly, next to the distinct Th lineage of Th9 cells, Th17 have also been described to be a source for IL-9. TGFβ and IL-1β were thereby identified as the main inducers of IL-9 and IL-17 co-expressing cells and mainly driving pathology in auto-immune diseases (11, 12).

A Th17 deficient immunological phenotype does not implicitly lead to altered allergic immune responses. The recently identified *gain-of-function* (GOF) mutation in STAT1 leads to deficient IL-17 production (13, 14). Patients with STAT1 GOF-mutation display next to recurrent infections and a higher incidence of auto-immune diseases or oesophageal cancer also chronic mucocutaneous candidiasis (CMC), manifesting as fungal infections on skin, nails and mucous membranes (14), but an atopic constitution is not a feature of the clinical presentation in these patients. In contrast, patients with the autosomal dominant *loss-of-function* (LOF) STAT3 mutation, also leading to a deficient IL-17 production, present with an atopic phenotype with eczema, eosinophilia and high IgE levels, as well as recurrent staphylococcal skin, pulmonary abscesses accompanied by bronchiectasis and also CMC (15, 16). In the present study we wanted to elucidate whether the clinical allergic phenotype in HIES correlates with alterations in Th2 and Th9 immune responses, and compare this with patients without allergic symptoms that share many features of HIES including a Th17 deficiency, namely CMC.

METHODS:

Volunteers and patients

Blood was collected from healthy volunteers or patients by venous blood puncture after informed consent. Healthy volunteers between 20 and 40 years of age with no chronic disease or allergic disease in their history as well as without any medication were asked for blood donation. Four CMC patients with an autosomal dominant mutation in the STAT1 gene donated blood, two of them twice at two different time points. Four patients diagnosed with HIES with an autosomal dominant mutation in the STAT3 gene were asked for blood donations; two of them donated blood twice at different time points (demographic characteristics, Table I). Four patients with atopic dermatitis (AD) recruited from the department of dermatology and diagnosed according to their clinical presentation were included into the study (demographic characteristics, Table II).

Stimuli, recombinant cytokines and blocking antibodies

Staphylococcus aureus isolated form a clinical case was cultured by the department of microbiology, heat killed and provided to us (*S. aureus*) ($1x10^7$ /ml).; *Candida albicans* ATCC MYA-3573 (UC820) yeast (*Ca yeast*) ($1x10^6$ /ml) were grown overnight in Sabouraud broth at 37˚C, cells were harvested by centrifugation, washed twice, and resuspended in culture medium. *Ca yeast* was heat-killed for one hour at 95 ˚C. The lack of viability was tested by culturing heat-killed yeast over night at 37˚C and used, when no growth was observed (17). *Aspergillus fumigatus* clinical isolate V05-27 (*Asp con*), resting conidia ($1x10^7$/ml) were cultured and isolated as described previously (18). Briefly, *A. fumigatus* was cultured on Sabouraud glucose agar supplemented with chloramphenicol for 7 days at 37˚C. Conidia were harvested by gently scraping the surface of the slants and suspending them in PBS with 0.05% Tween 80. To remove hyphae and debris, the conidial suspension was filtered through 4 layers of sterile gauze. The Tween of the mixture of conidia and PBS was removed by washing 3 times with PBS, resuspended into cell culture medium and adjusted to a concentration of $4x10^8$/ml. Conidia were heat-killed at 95˚C for 30 minutes. The lack of viability was checked by culture in Sabouraud glucose broth. Conidia were stored at -80˚C until used for the experiment.

Recombinant human IL-4 (10ng/ml), IL-1β (10ng/ml) and IL-23 (50ng/ml) were all purchased from R&D Systems. The anti-TGFβ I neutralizing antibody (10μg/ml) and its isotype control monoclonal IgG1 mouse anti-human antibody (10μg/ml) were purchased from R&D Systems.

Table I: *Demographic characteristics of CMC and HIES patients*

	Age at exp.	Gender	Serum IgE (IU/ml)	Eosinophil count ($x10^9$/l)	Clinicial presentation – allergic phenotype	Treatment	Mutation
CMC 1	70	male	< 2	0.02	Chronic mucocutaneous candidiasis, recurrent pulmonary infections, COPD, squamous cell carcinoma, auto-immune hemolytic anemia Allergies : no atopic eczema	Colistin, Anidulafungin, Azithromycin, Citalopram, Cidesonid, Calciumcarbonat, Amphotericin B, Prednisolon (10mg/d)	R274W
CMC 2	43	male	N/A	0.04	Chronic mucocutaneous candidiasis, recurrent pulmonary infections, bronchiectasis Allergies: no atopic eczema	Fluconazole, Amoxiclav	QT267R
CMC 3	31	male	< 2	0.20	Chronic mucocutaneous candidiasis, type I diabetes, hypothyreodism, recurrent pulmonary infections, SLE Allergies : no atopic eczema	Prednisolone (stop 5 month before testing), Itraconazole, Furosemid, Doxycycline, L-Thyroxin, Insulin	D23V
CMC 4	40	male	-	0.18	Chronic mucocutaneous candidiasis, recurrent pulmonary infections with abscess, recurrent sinusitis Allergies: none, no atopic eczema	Anidulafungin, Flucoxacillin, Ibuprofen	R274W
HIES 1	43	female	2702	0.21	Hyper IgE syndrome, recurrent staphylococcal skin abscesses Allergies: none, no atopic eczema	Miconazol, Flucloxacillin, Valciclovir	S560del
HIES 2	45	male	3942	0.14	Hyper IgE syndrome, recurrent pneumonia with *S. aureus*, bronchiectasis, pulmonary aspergilloma Allergies: hayfever, no atopic eczema	Moxifloxacin, Bacitracin, Ciprofloxacin, Amoxicillin, Miconazol, Doxicyclin	R382W
HIES 3	49	female	4524	0.13	Hyper IgE syndrome, recurrent staphylococcal and streptococcal upper airways infections, recurrent mucomycosis and skin abscesses, chronic mucoutaneous candidiasis, kidney abscess Allergies: none, no atopic eczema	Amoxiclav	R382Q
HIES 4	33	female	7493	0.33	Hyper IgE syndrome Allergies: none, atopic eczema	Flucloxacillin, Itraconazol	V473del

Table II: *Demographic characteristics of AD patients*

	Age at exp.	Gender	Serum IgE (IU/ml)	Eosinophil count (x10^9/l)	Clinicial presentation – allergic phenotype	Treatment
AD 1	30	Female	899	0.86	Atopic eczema	Oral: promethazine, Local: Cetomacrogol salve, Clobetason salve, coal tar, vaseline/paraffine
AD 2	28	Female	<4,7	N/A	Atopic eczema	Local: vaseline/paraffine
AD 3	28	Female	302	N/A	Atopic eczema	Local: vaseline/paraffine
AD 4	38	Female	69	N/A	Atopic eczema	Local: vaseline/paraffin, betamethasone, clobetasol, tacrolimus, coal tar

All experiments were performed and conducted in accordance to Good Clinical practice, the Declaration of Helsinki, and the approval of the Arnhem-Nijmegen Ethical Committee (nr.2010/104).

Peripheral blood mononuclear cell (PBMC) isolation

Venous blood was drawn in 10 ml EDTA tubes. The blood was diluted 1:1 with Phosphate Buffered Saline (PBS). Subsequently PBMCs were isolated using Ficoll-paque (GE healthcare, Zeist, The Netherlands) density gradient centrifugation, as described previously (19). The PBMCs layer was collected and washed twice in cold PBS. Cells were reconstituted in Iscove's modified Dulbecco's Medium (IMDM) (life technologies) supplemented with 10 µg/ml gentamicin, and 10 mM pyruvate (Gibco, life technologies). The cells were counted with a particle counter (Beckmann Coulter) and the concentration was adjusted to $1x10^7$ cells/ml.

PBMCs stimulation

PBMCs were plated in a 96-well plate (Corning) at a final concentration of $2.5x10^6$ /ml in an end-volume of 200 µl per well. All stimulations were performed in the presence of 10% human pooled serum. Cells were incubated at 37°C with 5% CO_2, after 7 days supernatants were collected and stored at -20°C.

Cytokine measurements

IL-5, IL-9, IL-13, IL-17, IL-22 and TGFβ were measured in the cell culture supernatants using commercial ELISA kits (IL-5, IL-13, IL-17, IL-22 and TGFβ: R&D Systems; IL-9: Biolegend) according to the instructions supplied by the manufacturer.

Intracellular staining and flowcytometry

After 7 days incubation in the presence of the pathogen with or without additional IL-4, cells were re-stimulated for 4-6 hours with PMA (50 ng/ml) (Sigma-Aldrich) ionomycin (1 µg/ml) (Sigma-Aldrich), in 200 µl of IMDM medium supplemented with 10 µg/ml gentamicin, and 10 mM pyruvate (Gibco) and 10% human serum in the presence of Golgiplug (BD Biosciences, Breda, the Netherlands). Cells were stained extracellular using PE-Cy7–conjugated anti-CD4 (ITK Diagnostics BV) and ECD–conjugated anti-CD45 (Beckman Coulter). Fixation and permeabilization was performed with Cytofix/Cytoperm solution (eBioscience) according to the instructions supplied by the manufacturer. Cells were stained intracellular with anti-IL-4 (PE conjugated, BD Pharmingen), anti-IL-17A (FITC conjugated, ITK Diagnostics BV) and anti-IL-9 (Alexa 647 conjugated, ITK Diagnostics BV). Regulatory T cells (T-regs) were stained with anti-CD25 (PE-Cy7 conjugated, eBioscience) and anti-Forkhead Box Protein (FOX) P3 (APC conjugated, eBioscience). Fixated cells were measured with a FC500 flowcytometer (Beckman Coulter) and the data were analyzed using CXP analysis software v2.2 (Beckman Coulter).

Statistical analysis

The Mann-Whitney-U test was used to detect differences between healthy controls and patients. The Wilcoxon-Signed Rank test was used to detect differences between isotype control and blocking antibody. A p-value of < 0.05 was considered statistically significant ($^{*}=p < 0.05$, $^{**} = p < 0.01$ and $^{***} = p < 0.001$). Graphs represent cumulative results of all performed experiments and are presented as mean ± standard error of the mean. Data were analyzed with GraphPad Prism v 5.0.

RESULTS

CMC and HIES patients have reduced IL-17A and IL-22 responses

Stimulation of PBMCs from CMC and HIES patients showed a significant defect in IL-17 and IL-22 production (Figure 1). CMC patients demonstrated significantly lower IL-17 and IL-22 production after stimulation with *Aspergillus, Candida* and *S. aureus*, while HIES patients still produced significant amounts of IL-17, especially after *S. aureus* stimulation. HIES patients produced significantly less IL-22 after stimulation with all different pathogens. To further validate this, flowcytometry analysis was performed and in both patient groups a lower frequency of IL-17+ CD4+ cells was observed.

Th2 response is deficient in CMC but not in HIES and AD patients

Next, we investigated whether the Th2 responses can be induced in STAT1 GOF or STAT3 LOF patients which both lack optimal Th17 responses. Stimulation with diseases-associated pathogens *Aspergillus, Candida* or *S. aureus* showed decreased IL-5 and IL-13 production in CMC patients (Figure 2A and B), but not in HIES patients. We did not explore classical mitogenic stimulation with aCD3/aCD28 because it did not induce IL-5 and IL-13 in healthy volunteers (data not shown). Since the induction of IL-5 and IL-13 was relatively low, stimulation in the presence of IL-4 was performed to shift the Th responses towards an allergic phenotype. CMC patients demonstrated a significant defect in IL-5 and IL-13 production, while cells isolated from HIES patients responded normally when stimulated with different pathogens in the presence of IL-4 (Figure 2 C and D). To explore Th2 responses in another patients group with allergic phenotype, we included another control group of patients with AD. Except for one outlier in the AD patients group, comparable IL-5 and IL-13 levels were measured in the cell culture supernatants of PBMCs of AD patients and healthy controls using the same stimulation assay (Figure 3).

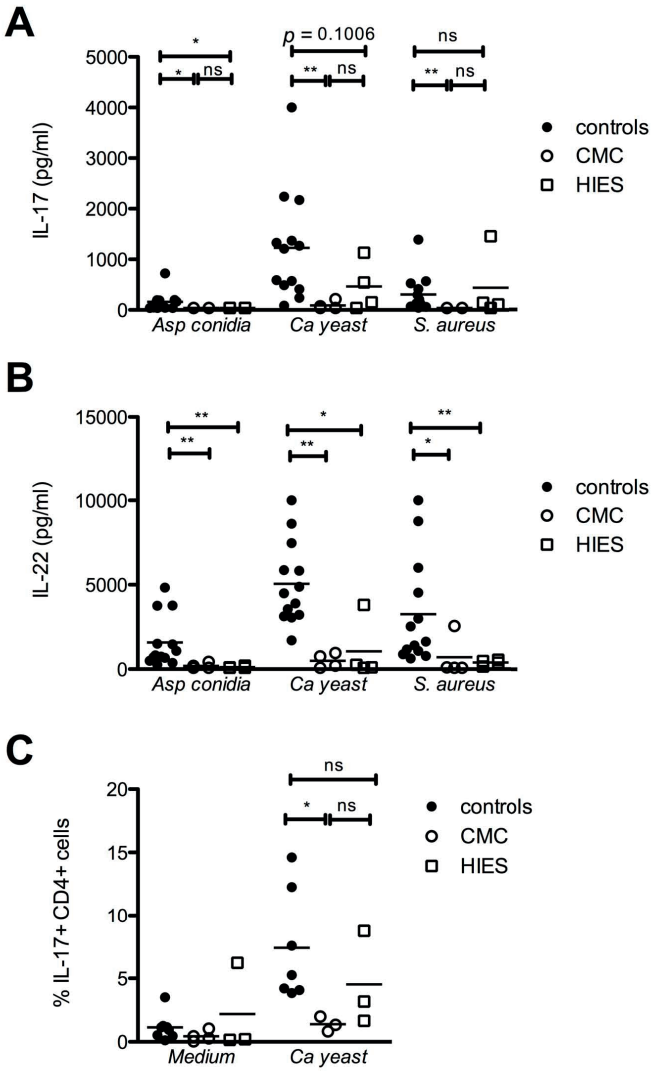

Figure 1: *CMC and HIES patients have reduced IL-17A and IL-22 responses*

PBMCs of healthy volunteers (n=13), CMC patients (n=4) and HIES patients (n=4) stimulated with heat-killed *Aspergillus* (Asp) conidia, *Candida* (Ca) yeast and *Staphylococcus* (S.) *aureus* and (A) IL-17 and (B) IL-22 measured in the cell-culture supernatant by ELISA or (C) stained for intracellular IL-17 and measured by flowcytometry (controls n=7, CMC n=2, HIES n=3). The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=p < 0.05, ** = p < 0.01 and *** = p < 0.001, ns = not significant).

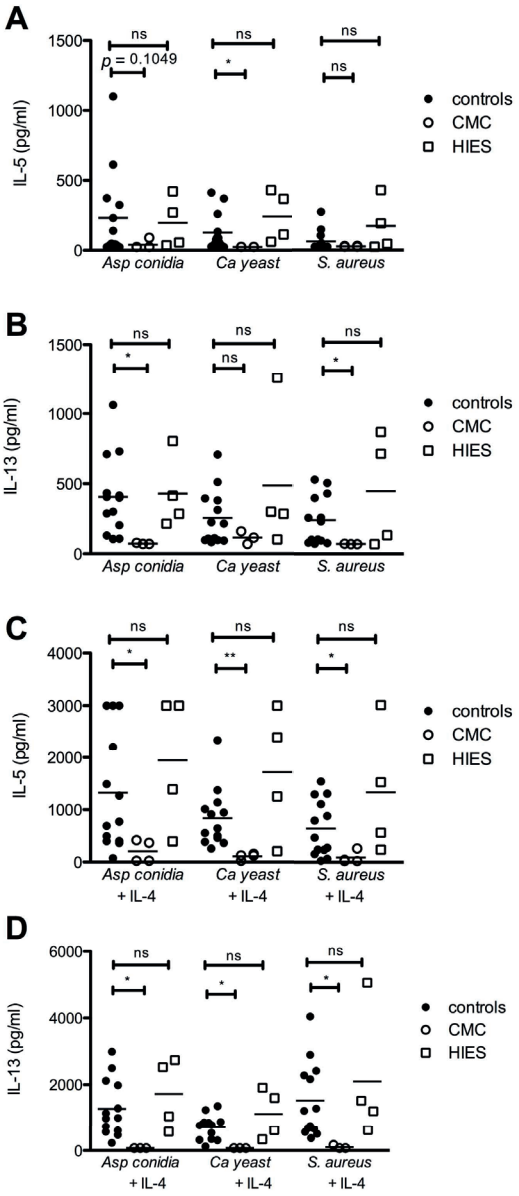

Figure 2: *The Th2 response of CMC patients but not of HIES patients is deficient*

PBMCs of healthy volunteers (n=13), CMC patients (n=4) and HIES patients (n=4) stimulated with heat-killed *Aspergillus* (Asp) conidia, Candida (Ca) yeast and Staphylococcus (S.) aureus (A+B) in the absence (C+D) or presence of IL-4. (A+C) IL-5 and (B+D) IL-13 measured in the cell-culture supernatant by ELISA. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$, ns = not significant).

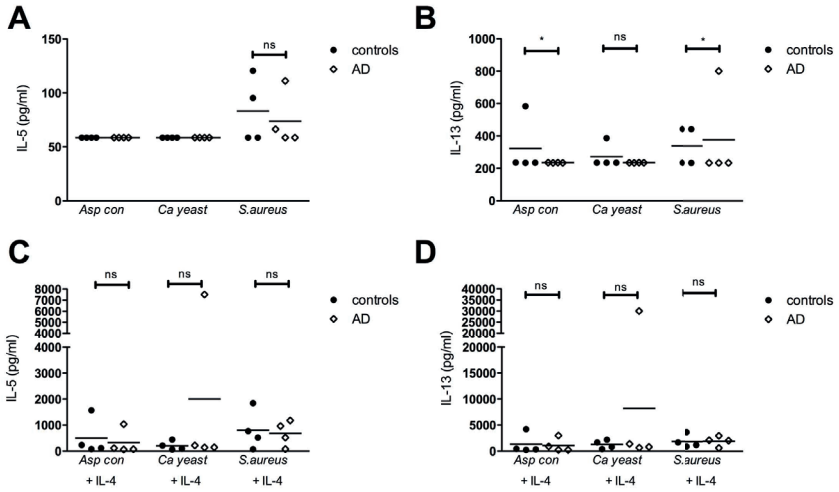

Figure 3: *The Th2 response of AD patients is comparable to healthy controls*

PBMCs of healthy volunteers (n=4) and AD patients (n=4) stimulated with heat-killed *Aspergillus (Asp)* conidia, *Candida (Ca)* yeast and *Staphylococcus (S.) aureus* (A+B) in the absence (C+D) or presence of IL-4. (A+C) IL-5 and (B+D) IL-13 measured in the cell-culture supernatant by ELISA. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=$p < 0.05$, ns = not significant).

IL-9 is induced by Candida and increased in the presence of IL-4 and IL-1/IL-23 in healthy controls

IL-9 has been described to be produced by Th17 cells under certain conditions; therefore we wanted to investigate IL-9 responses in patients with a defective Th17 response. Stimulation of PBMCs with different fungal pathogens or *S. aureus* demonstrated that *Candida* was the most potent inducer of IL-9 production (Figure 4A). The production increased steadily during stimulation for 7 days (Figure 4B). In the presence of the Th2-polarizing cytokine IL-4 and *Candida*, IL-9 production was further increased, while IL-4 stimulation alone did not induce detectable IL-9 production (Figure 4C). The IL-17-inducing cytokine cocktail IL-1β and IL-23 also increased *Candida*-induced IL-9 but to a lesser extend then IL-4 (Figure 4C). We next wanted to identify which T helper subset displayed IL-9$^+$ cells. *Candida* stimulation induced IL-9$^+$ cells, which were either single positive or present in the IL-17$^+$ subset. No IL-9$^+$ cells were detected in the IL-4$^+$ subset (Figure 4D). Since the frequency of IL-9$^+$ cells was very low, additional analysis was performed in the presence of IL-4 (Figure 4E). The percentage of IL-9$^+$ cells remained low even after IL-4 stimulation, and again IL-9 was almost absent in IL-4$^+$ cells, but present in the Th17 subset and IL-9 single positive cells.

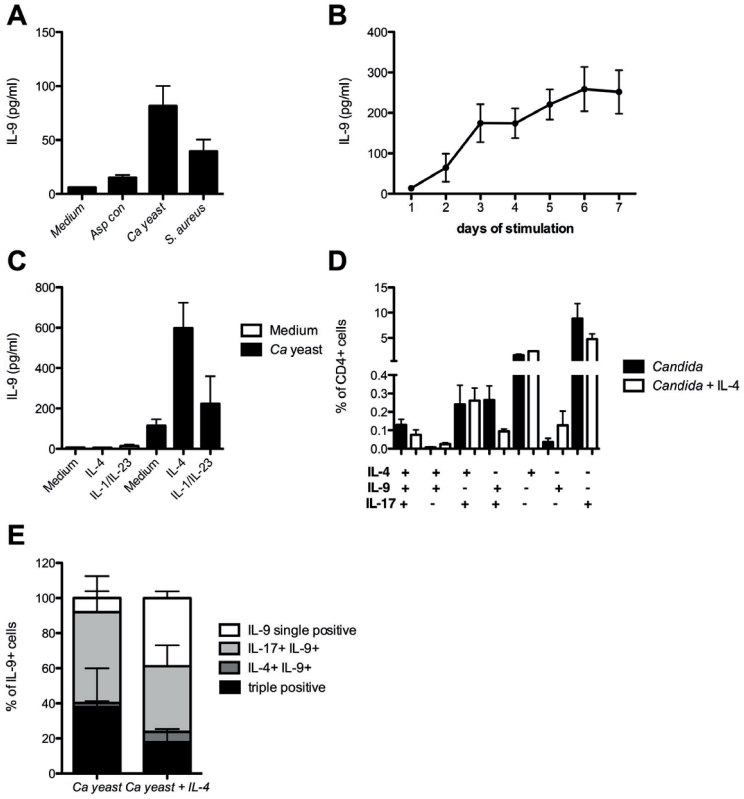

Figure 4: *IL-9 is induced by Candida and increased in the presence of IL-4*

PBMCs of healthy volunteers (n=6) stimulated with (A) different pathogens, (B) *Candida* yeast, or (C) *Candida* yeast in the presence of medium, IL-4or IL-1β/IL-23 . (A-C) IL-9-measured in the cell-culture supernatant by ELISA. (D+E) PBMCs of healthy volunteers stimulated with *Candida* yeast with and without IL-4. Intracellular cytokine combinations in percentage on all $CD4^+$ cells measured by flowcytometry.

HIES patients have a striking defect in IL-4 induced IL-9 production

Stimulation of PBMCs isolated from CMC and HIES patients resulted in significant lower *Candida*-specific IL-9 concentrations in CMC, while HIES cells produced IL-9, albeit lower (Figure 5). Interestingly, we observed that the addition of IL-4 to *Candida* stimulation resulted in significant lower IL-9 production in cells isolated from patients with HIES (Figure 5). Next, we measured IL-9 in the serum of HIES patients, AD patients and healthy controls; no IL-9 was detectable (data not shown).

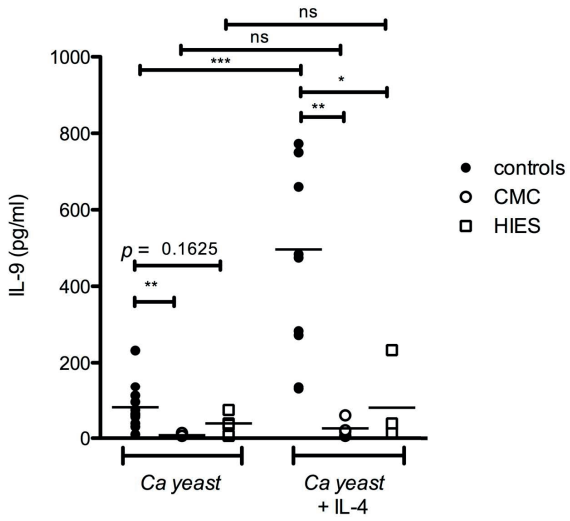

Figure 5: *CMC and HIES patients are deficient in IL-9 responses*

PBMCs of healthy volunteers (n=13), CMC patients (n=4) and HIES patients (n=4) stimulated with heat-killed *Aspergillus* conidia, *Candida* yeast and *S. aureus* in the absence or presence of IL-4 and IL-9-measurement in the cell-culture supernatant by ELISA. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$, ns = not significant).

This made us explore the underlying mechanism. Since TGFβ contributes to the induction of IL-9, and TGFβ expression of circulating activated T cells of HIES patients has been described to be low (20), we investigated whether TGFβ was responsible for this observed deficiency of IL-9 in HIES. The total IL-9 production was partially dependent on TGFβ in PBMCs of healthy controls. However, the synergistic effect of IL-4 on *Candida*-induced IL-9 was still present, when TGFβ was blocked in PBMCs of healthy controls (Figure 6A). In addition, TGFβ concentrations in cell culture supernatants of PBMCs isolated from HIES patients were comparable to healthy controls (Figure 6B).

TGFβ induces regulatory T cells (Tregs), which are able to modulate Th2 responses, therefore we measured the frequency of Tregs in HIES patients (21). The percentage of FOXP3+ CD25+ T-reg cells of total CD4+ cells was not different between healthy controls and HIES patients (Figure 6C). In contrast, intracellular staining with flowcytometry analysis of cells from HIES patients demonstrated a decreased percentage of overall CD4+ IL-9+ cells that was mainly due to a decrease in the IL-17+/IL-9+ cells compared to controls (Figure 6D).

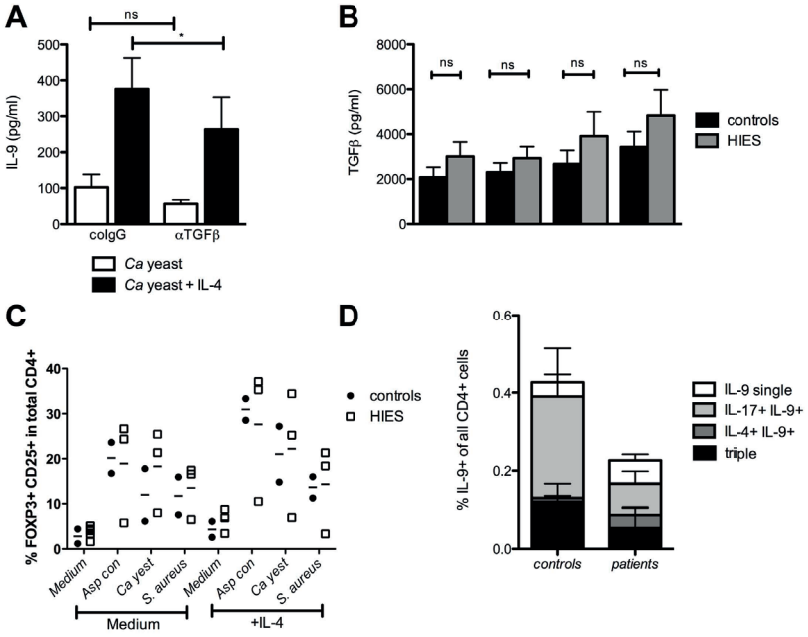

Figure 6: *HIES patients are IL-9 deficient not due to differences in TGF*β *production or T-regs, but due to a lower frequency of IL-17+ IL-9+ CD4+ cells*

(A) Blocking of TGFβ in PBMCs of healthy volunteers (n=6) stimulated with *Candida* yeast, with and without IL-4. (B) TGFβ-measurement in cell-culture supernatants of PBMCs of healthy controls (n=8) or HIES patients (n=4) stimulated with medium, *Aspergillus* conidia, *Candida* yeast and *S. aureus*. The Mann-Whitney-U test was used to determine, whether the means were significantly different (*=$p < 0.05$, ns = not significant). (C) T-reg-measurement by flowcytometry of healthy controls (n=2) or HIES patients (n=3) stimulated with medium, *Aspergillus* conidia, *Candida* yeast and *S. aureus* with and without IL-4. (D) Measurement of the intracellular cytokines IL-4, IL-9 and IL-17 of healthy controls (n=3) or HIES patients (n=3) by flowcytometry after stimulation with *Candida* yeast

DISCUSSION

The present study investigated Th2 and Th9 responses in patients with CMC and HIES. CMC patients had deficient Th2 response, while HIES patients were capable to produce IL-5 and IL-13, such as AD patients. Since both CMC and HIES patients are deficient in Th17 responses, this suggests that Th2 responses are initiated and regulated independently from IL-17. In addition, CMC patients were completely deficient on *Candida*-induced IL-9 production, with only a partial defect in HIES patients.

An interesting observation was a defect in IL-4-induced IL-9 production after stimulation with *Candida* in the HIES patients. IL-9 was co-expressed in a Th17 subset in healthy controls, and the percentages of this subset was lower in HIES patients, suggesting that

a robust IL-9 response is dependent on an intact Th17 subset that can produce IL-9. The intact Th2 response in HIES and a completely deficient Th2 response in CMC correlate with the clinical symptoms seen in CMC and HIES, because allergic responses are not a key feature of CMC, while atopic constitution with eczema, eosinophilia and high IgE levels are classical symptoms of HIES. Since we have not assessed the expression levels of IL-4R in CMC and HIES we cannot rule out that the IL-4R expression is significantly lower in CMC compared to HIES , which might explain our observations.

Th2 responses in HIES and CMC patients have been little studied until now. Here we describe a significant defect of IL-5 and IL-13 only in CMC patients, but not only after stimulation with Th2 inducing pathogens, but also after skewing these responses towards Th2 by the addition of IL-4. This is in line with a study investigating cytokine responses of eight CMC patients from Brazil, in whom no elevated Th2 responses were observed after mitogenic stimulation (22); it is important to note that in these patients the presence of STAT1 GOF mutations has not been tested. Khosravi *et al.* speculate about increased Th2 responses without having measured IL-4, IL-5 or IL-13 (23). However, Eyerich *et al.* had observed low interferon (IFN) γ and higher IL-10 in CMC patients and attributed the deficient Th responses to the elevated IL-10 response. One hypothesis is that STAT1 hyperphosphorylation (13) results in increased IFNγ signaling, subsequently leading to a strong inhibition of the Th17 differentiation resulting in an IL-17 production defect (24). This mechanism could also underlie the deficient IL-5, IL-13 and IL-9 production, since they are all inhibited by IFNγ (3, 25). One other explanation why all T helper subset are deficient in CMC would be a general defect in T helper cell proliferation. However, we observed that the T cell proliferation capacity of CMC patients was intact (Supplemental Figure 1), which is in line with previously published data (26). Nevertheless, a lower percentage of $CD4^+$ on $CD45^+$ cells as seen in CMC patients could also partially contribute to the low production of T helper derived cytokines (Supplemental Figure 2). In contrast, HIES patients displayed a selective IL-9 deficiency, while the Th2 cytokines IL-5 and IL-13 were produced in normal amounts. Intact Th2 responses were anticipated since HIES patients can present with an atopic constitution. AD patients presented with a robust Th2 response comparable to a non-allergic control group, suggesting that the Th2 assay used in this study is rather a qualitative assay than a quantitative assay for allergic responses. On the one hand, Boos *et al.* had described higher frequencies of Th2 cells in AD patients compared to healthy controls. On the other hand, they described comparable Th2 responses of HIES patients and healthy controls (27). Furthermore, these data are in line with previous reports describing normal frequencies of $CD4^+$ $IL\text{-}4^+$ cells, but elevated $CD4^+$ $IL\text{-}13^+$ cells (28), and normal Th2 responses (27, 29) in STAT3-HIES patients.

Collectively these data suggests that a disturbed immune balance causes the atopic pathology rather than a strictly isolated increased Th2 response.
Several studies suggest that T-regs, IL-10, and TGFβ are main players in regulating Th1/Th2 balance (30, 31). Interestingly, IL-10-treated dendritic cells derived from HIES patients have an impaired capability to induce FOXP3+ T-regs (31). However, we observed a normal frequency of peripheral T-regs in patients with HIES, which is in line with previously reported data (31). TGFβ is a cytokine of great interest in the context of our findings, since it is on the one hand a T-reg inducing cytokine (32), but in the presence of IL-4 it is a significant inducer of IL-9, and initiates the differentiation of naïve CD4+ T cells towards Th9 cells (33). Since we observed a deficient *Candida*-induced IL-9 response in the presence of IL-4 in HIES patients, we hypothesized that lower TGFβ concentration in HIES patients might be a possible mechanism. TGFβ concentrations were however not significantly different after stimulation in the patients. This is in line with the study of Saito *et al*., who also showed an intact TGFβ response in HIES patients (31), but in contrast with a study of Ohga *et al*., who described low TGFβ expression of circulating activated T cells of HIES patients (20). Interestingly, *Candida* induced more IL-9$^+$IL-17$^+$ co-expressing CD4$^+$ cells than IL-9$^+$ single positive cells in healthy volunteers. Secondary signals such as the IL-1 family cytokines IL-1α and IL-1β (34) have been described to potentiate IL-9 production. *Candida* is a strong IL-1 and IL-17 inducer (35), which is reflected by the relatively high frequency of IL-9$^+$IL-17$^+$ co-expressing CD4$^+$ cells in response to *Candida* compared to other stimuli. Here we show that HIES patients, most likely due to their deficient Th17 response due to their *STAT3* LOF mutation, also have a lower Th17 subset that is able to produce IL-9. Therefore it is likely that the lower IL-9 response in response to IL-4 in HIES patients is due to a deficiency to induce a subset of Th17 cells that are able to produce IL-9.

Since HIES patients suffer from cold staphylococcal abscesses, chronic mucocutaneous candida infection as well as from pulmonary aspergillosis, mainly in preformed bronchiectatic cavities causing aspergilloma or even invasive pulmonary aspergillosis, specific immune responses against these three pathogens have been investigated and compared to CMC patients (36). Interestingly, both fungal pathogens *Candida* and *Aspergillus* did not cause different immune responses, although *Candida* had a stronger cytokine inducing capacity. This matches with their clinical presentation, since it has recently been published that also CMC patients can suffer from *Aspergillus* and *S. aureus* infections (37) making HIES and CMC syndromes very similar in terms of susceptibility to fungal infections due to their Th17 deficiency.

In conclusion, CMC and HIES patients share several immunological defects, but differ in their capability to induce Th2 and Th9 responses. CMC patients have a completely

abolished Th2 and IL-9 response, while HIES patients have normal Th2 responses and only lack an IL-4-induced IL-9 response. These data might explain both the similarities and differences in clinical presentation seen between HIES and CMC patients; sharing an increased susceptibility to mucocutaneous candidiasis due to a Th17 deficiencies on the one hand, but displaying clear differences in allergic disease due to difference in Th2 and Th9 responses on the other hand. Why CMC patients present with a more general defect in Th subsets, and how the lack of IL-9 further contributes to the immunopathology of HIES patients remains to be elucidated.

ADDITIONAL MATERIAL: METHODS FOR SUPPLEMENTAL FIGURES:

CFSE proliferation assay

Proliferation of PBMCs was measured using the CFSE kit (Biolegend) according to the instructions described by the manufacturer. Briefly, PBMCs were re-suspended to a density of 10 x 10^6 cells/ml in PBS and labeled by adding CFSE in a 1:1 ratio to a final concentration of 1.25uM. The suspension was mixed gently and incubated for 5 minutes at 37˚C. An equal volume of 100% human pooled serum was added and incubated for 3 minutes at room temperature. Cells were washed two times in RPMI+ (RPMI-1640 culture medium - Dutch modification, Gibco, Invitrogen, Breda, The Netherlands - supplemented with 10 µg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate - Gibco) supplemented with 10% human pooled serum and re-suspended to a concentration of 5x10^6/ml in RPMI+. An unlabeled cell fraction and a labeled cell fraction were measured by flowcytometry on day 0 to control for the staining efficacy. Labeled cells were stimulated for 6 days with IL-2 (75 U/ml, R&D systems) and different pathogens in the presence or absence of IL-4. On day 6 cells were stained with anti-CD3 (PE-Cy5 conjugated, ITK Diagnostics BV) and measured by flowcytometry. The percentage of proliferated CD3+ cells were calculated as percentage of all CD3+ cells.

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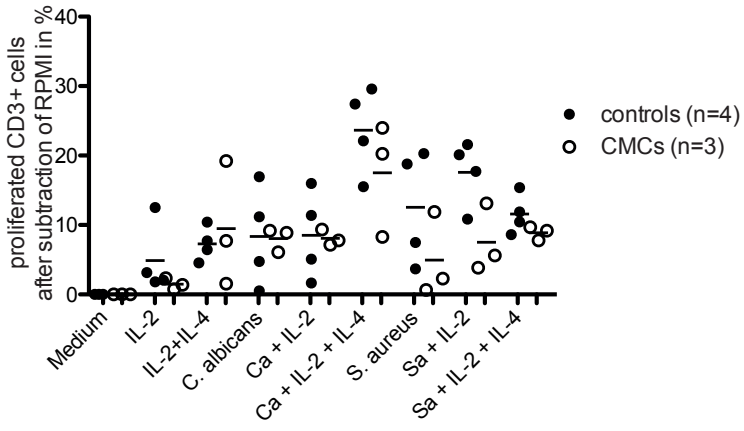
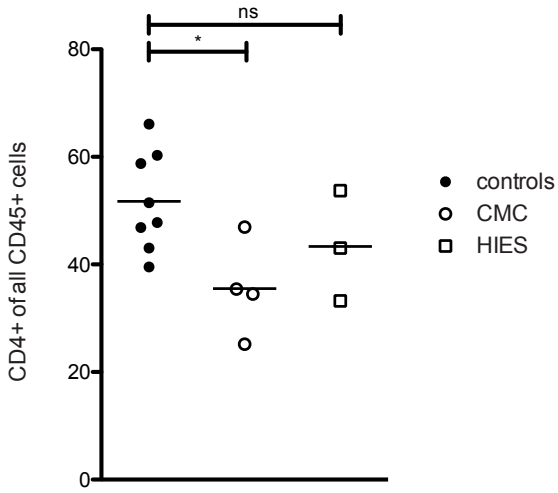


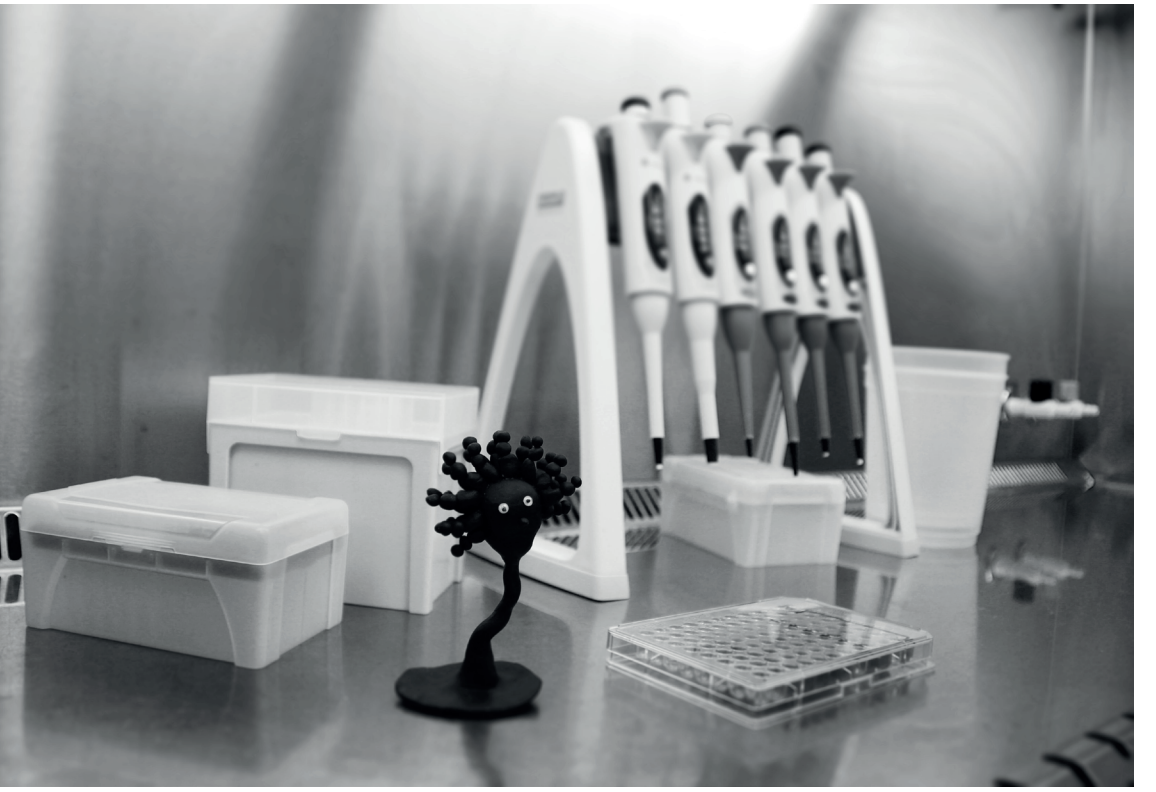
Supplemental Figure 1: *Proliferation assay of CMC patients*

Proliferation assay was performed after 7-days stimulation of CFSE-labelled PBMCs with IL-2 and *Candida* yeast or *S. aureus* with and without IL-4 of healthy controls (n=4) and CMC patients (n=3).



Supplemental Figure 2: *Frequency of CD4+ cells of CMC and HIES patients*

The percentage of $CD4^+$ cells within the $CD45^+$ cells was calculated in PBMCs of healthy controls (n=7) and patients with CMC (n =4) or HIES (n=3) after measurement by flowcytometry.



CHAPTER 10

Deficient interleukin-17 production in response to *Mycobacterium abscessus* in cystic fibrosis

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To the editor

The respiratory tract of patients with cystic fibrosis (CF) is colonized with a high diversity of microorganisms. Non-tuberculous-mycobacteria (NTM) show a high and increasing prevalence. Forty percent of these positive NTM cultures were caused by *Mycobacterium abscessus* (1), one of the rapidly growing NTM present in the environment. Patients with *M. abscessus* infection are difficult to treat due to natural and acquired antibiotic resistance (2, 3) and an infection with *M. abscessus* is controversially discussed as contra-indication for lung transplantation (4).

Immune-modulatory treatment strategies might contribute to overcome this problem. For their development, a better understanding of the defective immune response explaining the higher susceptibility of CF patients to *M. abscessus* is needed. Here we present three CF patients with *M. abscessus* infection, in whom we describe the pathogen-specific innate and adaptive cytokine production and compare this with non-CF patients with pulmonary infection caused by various NTMs: *M. abscessus* (n=1), *M. avium* (n=3), *M. kansasii* (n=2), *M. intracellulare* (n=1).

Case 1 is a 24-year-old female patient with CF (dF508del/dF508del) with pancreatic insufficiency and *Pseudomonas* colonization since 2003. In 2004, she presented with allergic bronchopulmonary aspergillosis (ABPA) that was successfully treated with corticosteroids. After years of infectious exacerbations she presented with an episode of haemoptysis in 2010. Shortly thereafter, *M. abscessus* was cultured from her sputum. In 2011, haemoptysis and clinical deterioration led to hospitalisation and several courses of antimycobacterial regimens (combinations of amikacine, clarithromycin, tigecycline, meropenem and clofazimine) were given without successful *M. abscessus* eradication.

Case 2 is a 23-year-old male patient with CF (dF508del/dF508del), pancreatic insufficiency and *Staphylococcus aureus* and *Aspergillus* colonization presented with ABPA. After a course of corticosteroids and itraconazole, he improved and serological markers for ABPA have remained at low levels ever since. After this episode, *M. abscessus* was consistently cultured and although he had no physical complaints, his pulmonary function deteriorated, and a CT thorax showed several sub-pleural and intra-parenchymatous nodular lesions compatible with mycobacterial disease. No clearance of *M. abscessus* was achieved, despite two courses of treatment with combination regimens of amikacin, meropenem, clarithromycin and clofazimine.

Case 3 is a 15-year-old male patient with CF (dF508del/G551D), pancreatic insufficiency and *Pseudomonas* colonization. Since 2011 *M. abscessus* was consistently cultured and he experienced several exacerbations in 2014 in which his pulmonary function deteriorated, despite several NTM regimens (including tigecycline, clofazimine, linezolid, azithromycin) and treatement with ivacaftor. However, he was non-compliant for the

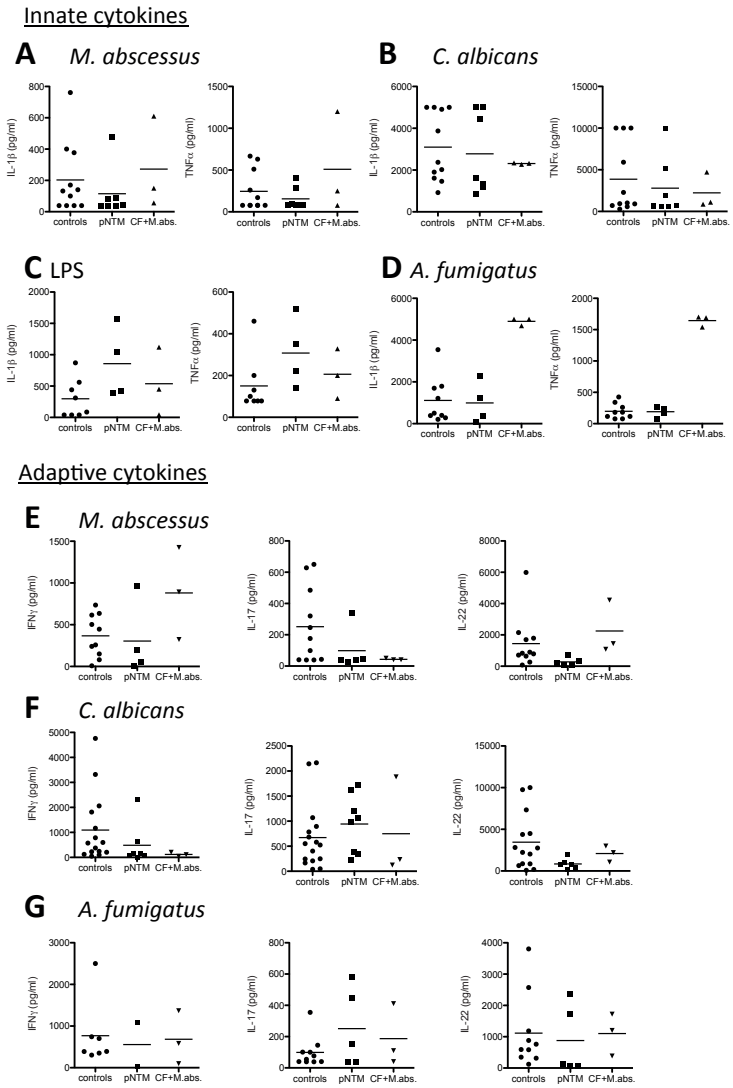

Figure 1: *Innate and adaptive cytokine responses of three CF patients with M. abscessus infection compared with non-CF patients with pulmonary NTM infection.*

PBMCs (2.5x10^6/ml) of a healthy control group (n = 11), non-CF patients with pulmonary NTM infections (pNTM) (n=7) and three CF patients with *M. abscessus* infection were stimulated with heat killed *M. abscessus* (clinical isolate NLA001001395) (1x10^5/ml), heat killed *C. albicans* (ATCC MYA-3573, UC820) (1x10^5/ml), *E.coli* LPS (*E. coli* serotype O55:B5, Sigma-Aldrich St. Louis, MO USA) (1ng/ml) or *A. fumigatus* conidia (clinical isolate V05-27) (1x10^7/ml). The innate cytokines TNFα and IL-1β were measured after 24h and the adaptive cytokines IFNγ, IL-17.

treatment and not able to complete several regimens due to side effects and complex psychosocial situation. No relevant clearance of *M. abscessus* was achieved.

PBMCs isolated from the three patients described above were stimulated with *M. abscessus,* isolated and cultured from the second case, *C. albicans, E. coli* LPS and *A. fumigatus*. CF patients and pulmonary NTM patients did not demonstrate a distinct innate cytokine profile after PBMC stimulation with *M. abscessus* or *C. albicans* (Figure 1 A+B). Interestingly, all NTM patients showed higher TNFα and IL-1β production on LPS stimulation compared to the control group, while the *A. fumigatus*-specific innate immune response was selectively increased in the CF patients infected with *M. abscessus* (Figure 1 C+D).

CFTR-deficient monocytes have been described to be highly reactive to LPS stimulation due to a prolonged sensing period of TLR4 on the cell surface (5). Furthermore, previous studies reported a hyper-responsive innate immune system in CF patients, with elevated TNFα, IL-1β and IL-6 levels in the sputum (6), which is in line with the elevated innate immune profile described in the CF patients of our study. CF patients who are often colonized with *Aspergillus* are on high risk to develop allergic bronchopulmonary aspergillos (ABPA) (7.8%) (7), and two out of the three CF patients with NTM infection in this study fulfilled the criteria of ABPA. Whether and how the strongly increased innate cytokine response after *Aspergillus* stimulation as seen in all CF patients in this study contributes to the development of ABPA or is the result of a hypersensitivity reaction in an *Aspergillus* colonized lung remains to be elucidated.

By contrast, the acquired immune responses revealed pathogen-specific differences. To investigate adaptive T helper (Th) cell responses, PBMC were stimulated with *M. abscessus, C. albicans,* which was used as a positive control for IL-17 production, and *Aspergillus* conidia. PBMCs from healthy subjects produced IL-17, IL-22 and IFNγ in response to *M. abscessus* (Figure 1E). Although PBMCs isolated from both patient groups were capable of producing IL-17 in response to *C. albicans* (Figure 1F), IL-17 in response to *M. abscessus* was very low in all CF patients as well as in most of the patients with pulmonary NTM infection. By contrast, *M. abscessus* induced IL-22, and IFNγ was similar or even elevated in CF patients infected with *M. abscessus*, while the pulmonary NTM patients demonstrated low IFNγ and IL-22 production on *M. abscessus*-stimulation. This suggests a selective pathogen-specific immunodeficiency in CF patients infected with *M. abscessus* shown by the total absence of *M. abscessus*-specific IL-17 production. Interestingly the *Aspergillus*-specific T helper (Th) 1 and 17 responses did not show differences between the groups.

CF is associated with a high prevalence of *M. abscessus* infections (1), but the underlying host defects leading to this increased susceptibility are not fully understood. Abnor-

malities in the IL-12/IFNγ-signalling pathways are a known risk factor for mycobacterial infections (8) and decreased IL-17 production in response to *Mycobacterium avium* has been described in PBMCs isolated from patients with NTM lung disease (9). This is in line with the low T helper cytokine deficiency of the pulmonary NTM patients in this study. The Th17 subpopulation has been described to play a protective role in mucosal immunity against extracellular fungal and bacterial infections, especially in the lung (10). Although some of the healthy volunteers responded only poorly on *M. abscessus*-stimulation, in most donors a robust Th17 response was induced as described earlier (11). The three CF patients in this study demonstrated a *M. abscessus*-specific IL-17 deficiency, while the cells were not intrinsically deficient in IL-17 production. Despite earlier reports that described significantly elevated IL-17 level in sputum and airway mucosa of CF patients, we propose that *M. abscessus*-specific IL-17 deficiency can promote the susceptibility to chronic NTM infections (12).

Treatment with biological drugs blocking innate cytokines that promote Th17 differentiation is associated with an increased risk of NTM infections (13). Therefore we propose the inverse correlation that patients with low IL-17-responses, such as the three CF patients in this study, might profit from an IL-17 supporting treatment regimen. Only a few studies has been performed to date. Next to treatment with IFNγ, which had beneficial effects on the IL-17 and IL-22 production in patients with severe fungal infection (14), one might speculate about the supplementation of recombinant IL-17. Low-dose IL-17 has recently been shown to be prevent diabetic nephropathy and organ fibrosis (15).

In conclusion, we describe pathogen-specific cytokine signatures in three CF patients and *M. abscessus* infection, two of them with ABPA, which might contribute to their higher susceptibility of NTM and *Aspergillus*-specific responses. The *M. abscessus*-specific IL-17 deficiency rather than an IFNγ-defect might play a crucial role in promoting pulmonary non-tuberculous mycobacterial infections in CF. This might have direct implementation for different immune-modulatory treatment regimens in pulmonary NTM infection in patients either with CF or without.

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CHAPTER 11

MST1R mutation as a genetic cause of Lady Windermere syndrome

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To the editor

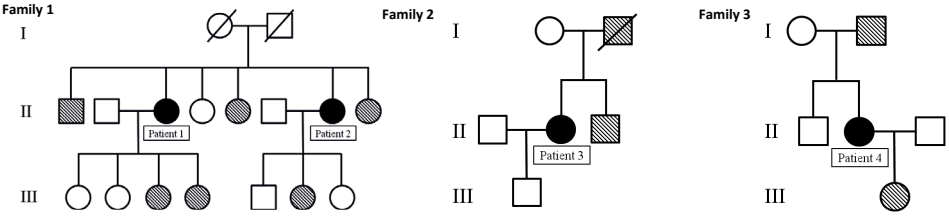
The prevalence of pulmonary NTM (pNTM) disease is increasing (1). The most commonly isolated NTM to cause disease belong to the *M. avium* complex (MAC) (1). Susceptibility to and clinical manifestation of NTM disease is largely governed by the immune status of a person. Disseminated or extrapulmonary NTM infections are strongly associated with severe immunosuppression such as those with frank defects in the interferon-gamma (IFNγ)-interleukin-12 (IL-12) axis (2). Isolated pNTM is strongly associated with certain underlying conditions, such as cystic fibrosis, chronic obstructive pulmonary disease, and primary ciliary dyskinesia (3, 4). However, substantial number of pNTM patients have no apparent risk factors and a significant proportion of them exhibit a body morphotype characterized by life-long slender body habitus, pectus excavatum (PEX), scoliosis, and mitral valve prolapse (5, 6), also called the lady Windermere syndrome. Patients were described to have a modest reduction in IFNγ production and an increase in transforming growth factor-beta (TGFβ) levels (7-10). Fowler and co-workers quantified ciliary beat frequency of 58 pNTM patients and 40 controls and found reduced ciliary beat frequency in the pNTM patients (11). Szymanski *et al.* recently performed whole exome sequencing on patients with pNTM, their unaffected family members and a control group and concluded that pNTM is a multigenic disease, encompassing potential defects in proteins encoded by cilia genes, the *cystic fibrosis transmembrane conductance regulator* gene, connective tissue genes, and certain immune-related genes (12).

Since PEX and scoliosis have been described in several genetic disorders that are not necessarily notable for increased susceptibility to lung infections, it suggests that pNTM patients with this body phenotype may have a more specific genetic basis that could not only account for their increased vulnerability to pulmonary NTM infections (13). The goal was to determine the genetic basis for patients with lady Windermere syndrome, namely the pNTM patients with PEX and scoliosis ($pNTM^{PEX/scoliosis}$) by performing whole exome sequencing in 11 individuals with this phenotype and functional validation of the genetic findings.

Eleven individuals with $pNTM^{PEX/scoliosis}$ – one family with two sisters and nine sporadic cases – were recruited from National Jewish Health and University of Colorado Anschutz Medical Campus, respectively, as part of a previous study (5). In whole exome sequencing *MST1R* was the only gene with a previously described role in innate immunity or cilia function that harbored rare non-synonymous variants in 3 families.

The two sisters (Patients 1 and 2 in Family 1, Figure 1A) carried a very rare missense variant (p.V900M) in *MST1R* with a very low population frequency of 0.0004794 or 28/58,406 public exomes based on Exome Aggregation Consortium (ExAC) (14). Co-segregation analysis in their extended family revealed that three siblings and three children are

(A)

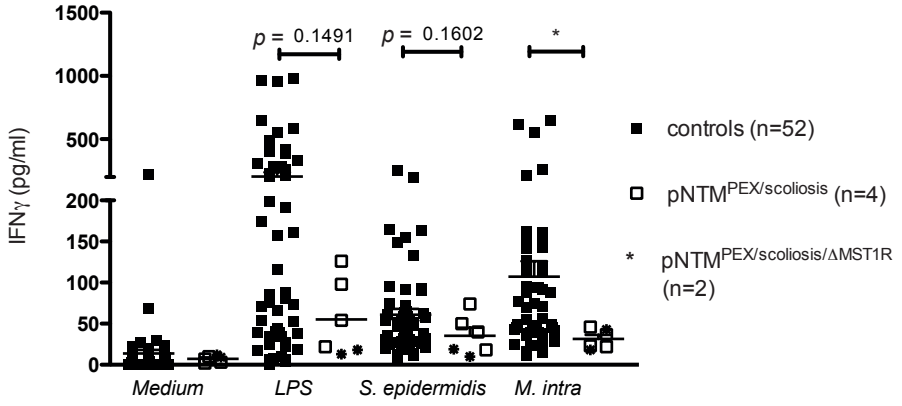


Family 1: patients 1 & 2		
Individual	**Complaints**	**MST1R mutation**
I.I	Unknown	DNA N/A
I.II	Unknown	DNA N/A
II.I	Pectus excavatum, occasional pneumonia, throat clearing with phlegm	V900M/wt
II.II (Patient 1)	**pNTM, pectus excavatum and scoliosis**	**V900M/wt**
II.III	unaffected	wt/wt
II.IV	Chronic cough	V900M/wt
II.V (Patient 2)	**pNTM, pectus excavatum and scoliosis**	**V900M/wt**
II.VI	Unaffected	V900M/wt
III.I	Unaffected	wt/wt
III.II	Unaffected	wt/wt
III.III	Unaffected	V900M/wt
III.IV	Unaffected	V900M/wt
III.V	Unaffected	wt/wt
III.VI	Unaffected	V900M/wt
III.VII	Unaffected	wt/wt

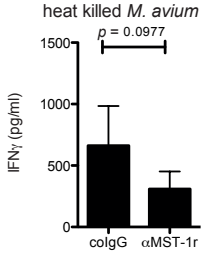
Family 2: patient 3		
Individual	**Complaints**	**MST1R mutation**
I.I (Mother)	Unaffected	wt/wt
I.II (Father)	Frequent severe bronchitis, throat clearing with phlegm	DNA N/A (p.M1383T/wt)*
II.I (Patient 3)	**pNTM, pectus excavatum and scoliosis**	**p.M1383T/wt**
II.II	Pneumonia, susceptible to chest infections; throat clearing	p.M1383T/wt
III.I	Unaffected	DNA N/A

Family 3: patient 4		
Individual	**Complaints**	**MST1R mutation**
I.I (Mother)	Unaffected	wt/wt
I.II (Father)	Pronounced cough >20y , raspy hoarse voice	p.D176N/wt
II.I	Unaffected	wt/wt
II.II (Patient 4)	**pNTM, pectus excavatum and scoliosis**	**p.D176N/wt**
III.I	Unaffected	p.D176N/wt

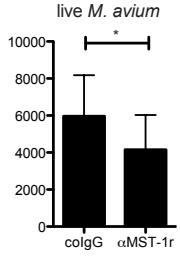
(B)



(C)



(D)



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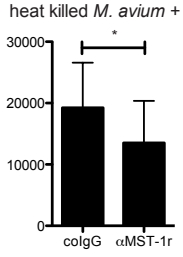

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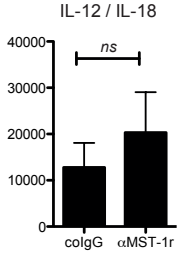

Figure 1: (A) Family 1: The two sisters with pNTM$^{PEX/scoliosis}$ carry a mutation in *MST1R*. Three of their siblings also have DMST1R and two of them have chronic respiratory symptoms. Family 2 The index patient with pNTM$^{PEX/scoliosis}$ has DMST1R. One brother of the index patient also has the same *MST1R* mutation and has frequent chest infections. *Because two of his children carry the *MST1R* mutation, the deceased father of the family is the expected carrier of the mutation, although his DNA was not available. Family 3 The index patient with pNTM$^{PEX/scoliosis}$ has DMST1R. Both the father and daughter of the index patient also carry the MST1R mutation but only the father has a chronic cough whereas the daughter has no health issues or complaints. (B) Whole blood of healthy controls (black squares), patients with pNTM$^{PEX/scoliosis}$ (open squares) and pNTM$^{PEX/scoliosis}$ patients carrying the MST1R mutation (pNTM$^{PEX/scoliosis/\ \Delta MST1R}$, open circles) was stimulated with a medium control, LPS, heat-killed *S. aureus* and *M. avium*. IFNγ was measured in the supernatant by ELISA. The Mann-Whitney-U test was used to determine, whether the means were significantly different. (C-F) PBMCs of healthy donors were stimulated for 48 hours with (C) heat killed *M. avium* (n=6), (D) live *M. avium* (n=6), (E) heat killed *M. avium* + IL-12 (n=6), or (F) IL-12/IL-18 (n=7) in the presence of a MST1R neutralizing antibody (γMST1R) or its isotype control. IFNγ was measured in the cell culture supernatant by ELISA. The Wilcoxon Signed Rank test was used to determine whether the mean values were significantly different.

carriers of the same *MST1R* variant (Figure 1A). Two of the nine sporadic pNTM$^{PEX/scoliosis}$ cases also have missense variants in the *MST1R* gene. Patient 3 had a private missense variant not previously reported (p.M1383T) and patient 4 possessed a rare missense variant (p.D176N) with a very low population frequency of 0.0009082 or 53/58,360 public exomes (14). Co-segregation analyses within the respective families showed that in both Patients 3 and 4 of Family 2 and 3, the *MST1R* variant was paternally inherited (Figure 1A). Sanger sequencing revealed that all 29 pNTM patients without PEX or scoliosis were negative for rare *MST1R* variants.

We next addressed cytokine production of pNTM$^{PEX/scoliosis}$ patients. Whole blood stimulation experiments revealed significantly lower *M. intracellulare*-induced IFNγ production in all pNTM patients compared to the control group as previously reported (5) (Figure 1B). IL-10 production in the pNTM$^{PEX/scoliosis}$ patients was also lower after stimulation with LPS, *S. epidermidis,* or *M. intracellulare* (data not shown). The mean concentrations of IL-6 were not significantly different between pNTM$^{PEX/scoliosis}$ patients and controls (data not shown). In contrast, the anti-inflammatory cytokine TGFβ was significantly increased in pNTM$^{PEX/scoliosis}$ patients compared to controls (data not shown), also shown in a recent study (7). Comparing pNTM$^{PEX/scoliosis}$ patients with and without the variant in *MST1R* revealed as low or even lower levels of IFNγ in whole blood stimulation (Figure 1B).

To investigate the immunological relevance of *MST1R*, we stimulated PBMCs from healthy individuals with heat-killed *M. avium,* live *M. avium*, heat-killed *M. avium* + IL-12, or IL-12 + IL-18 in the absence or presence of a MST1R neutralizing antibody, and measured IFNγ levels (Figure 1D-F). Blocking MST1R resulted in a significantly lower level of IFNγ in

response to live *M. avium* ± IL-12 and a trend toward reduced IFNγ production with heat-killed *M. avium* (Figure 1C-E). However, with IL-12+IL-18 stimulation of the PBMCs, there was no difference in IFNγ production with or without anti-MST1R antibody (Figure 1F). Furthermore, blocking MST1R had no effect on *M. avium* (heat-killed or live) stimulation of IL-10, IL-6, or TGFβ by the PBMCs (data not shown). This reveals a previously undescribed potential mechanism by which MST1R plays a host defense role against NTM.

Interestingly, MST1R variants were also recently reported in three pNTM patients in a cohort of 77 NTM patients (12). While this is consistent with our findings, it is interesting to speculate that the higher frequency of *MST1R* variants in our smaller cohort may be due to more stringent selection of patients with both PEX and scoliosis. To evaluate this possibility, we performed Sanger sequencing in 29 patients without these physical characteristics and found no rare genetic variation in *MST1R*. How *MST1R* variants might be related to the presence of PEX/scoliosis is unknown; furthermore, because PEX/scoliosis are also present in some patients without MST1R mutation raises the strong possibility that PEX/scoliosis are associated with anomalies of more than one gene. Indeed, this notion is supported by the finding that PEX/scoliosis is not only seen with Marfan syndrome but also several other connective tissue disorders such as Loeys-Dietz and Shprintzen-Goldberg syndromes (13). Interestingly, all these connective tissue disorders are due to increased TGFβ signaling albeit through different mechanisms.

MST1R is highly expressed on airway epithelial cells and functions to increase mucociliary function; *i.e.*, binding and activation of MST1R by its ligand macrophage-stimulated protein (MSP) leads to a significant increase of the ciliary beat frequency (15). Thus, a defect in the mucociliary transport due to a *MST1R* mutation could lead to impaired clearance of NTM, resulting in a vicious cycle of airway inflammation and infection leading to the bronchiectasis typically seen in patients with pNTM. These genetic findings are consistent with the study showing that adult patients with pNTM and without known predisposing factors have reduced ciliary beat frequency (11). Thus, defects in MST1R function can specifically attenuate IFNγ production as well as decrease airway ciliary function, with both defects increasing susceptibility to pNTM. The decrease in IFNγ production was specific for the stimulation with live *M. avium*, since blocking MST1R did not decrease IL-12 + IL-18-induced IFNγ production. The reason for this phenomenon is not known; it might be speculated that *M. avium* has a direct ligand for MST1R.

In conclusion, we have identified rare variants in MST1R in four of 11 pNTM$^{PEX/scoliosis}$ patients, and suggest that these genetic variants contribute to lady windermere syndrome by decreasing airway ciliary function and reducing IFNγ production in response to NTM.

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CHAPTER 12

Summary and Conclusions

Fungi are environmental microorganisms that make a valuable contribution to the ecosystem. Humans are in permanent contact with fungi and a healthy immune system can deal with the fungal contact easily: either by tolerating commensals such as *Candida* which resides on the human mucosal surface or by clearing fungal pathogens such as airborne *Aspergillus*, of which we inhale more than several hundred spores on a daily basis without developing any signs of illness. However in patients lacking efficient defence mechanisms the fungus can invade the tissue and cause disease. While chemotherapy is the most common cause for a severe immunodeficient status of humans, many genetic defects have been identified over the past decades that also play a key role. This ranges from single nucleotide polymorphisms being associated with a higher susceptibility for fungal infections under immunocompromised conditions, to monogenetic mutations leading to severe fungal diseases. In contrast, a hyper-reactive immune system triggered by fungi can cause allergic disease. *Aspergillus* has a potent allergenic capacity and can cause allergic bronchopulmonary aspergillosis, especially in patients with severe asthma or cystic fibrosis. Treatment options are still limited for both invasive and allergic fungal disease and a better understanding of the molecular mechanisms underlying the antifungal host response is the basis for the development of novel target-specific and immunomodulatory treatment regimens.

ASPERGILLUS RECOGNITION AND INITIATION OF T HELPER RESPONSES

Studying fungal immunology always has totake into account both sides, the fungus and the host. Different morphologies of the fungus will lead to the exposure of a fundamentally different range of associated molecular patterns. The first step of an efficient innate immune response is the recognition of these different structures by pattern recognition receptors that subsequently initiate a strong adaptive immune response [1]. In **Chapter 2** I present an overview of the range of pattern recognition receptors recognizing the two most commonly isolated pathogenic fungi, namely *Aspergillus fumigatus* and *Candida albicans*. Although only a certain number of the pattern recognition receptors and downstream signalling cascades are described for the antifungal host response, the crosstalk and conditional interaction between these pathways allow the immune system to establish a tailored innate and adaptive immune response [2]. The synergistic effects of Dectin-1 and TLR2, the activation of the required dectin-1-mediated phagocytosis and signals and the subsequent activation of the NLRP3 inflammasome, are some of the most important innate immune mechanisms responsible for the antifungal immune response [3, 4]. A complex interplay of different immune cells ranging from neutrophils,

monocytes, macrophages, NK-cells, T-cells to innate lymphoid cells and even epithelial and endothelial cells orchestrate the anti-fungal immune response.

Polysaccharides of the fungal cell wall serve as receptor ligands for PRRs. Wer performed a set of studies to elucidate their corresponding receptors in order to better understand the molecular mechanism of fungal recognition. In **Chapter 3** we investigated immunomodulatory properties of the *Aspergillus* cell wall component chitin. Different immune functions have been attributed to chitin to date: while Bueter *et al*. described chitin as an inert particle, Wagener *et al.* observed a strong anti-inflammatory capacity of chitin mediated by mannose receptor, TLR9 and NOD2 [5, 6]. Interestingly, the variation inpurity, size, shape, preparation and experimental setup can explain the important differences between the results described in the literature [7]. In this study we used *Aspergillus* chitin with a high grade of acetylation and a particle size of < 0,5µm, proceeded by short time ultrasonication; we ensured high purity due to removing remaining β-glucan by β-glucanase treatment. By stimulating human PBMCs in the presence of human serum we identified the Fcγ/Syk/PI3K pathway, through which chitin can induce strong anti- and pro-inflammatory immune responses. We also described that the *Aspergillus* cell wall component chitin is a trigger of a potent anti-flammatory response by inducing IL-1Ra in human PBMCs, while no other pro-inflammatory cytokine was induced by chitin. We identified immunoglobulins in human serum as necessary opsonins to activate the Fcγ/Syk/PI3K pathway, which is the key pathway involved in chitin-specific IL-1Ra response, while the chitin particles themselves were immunologically inert. In contrast, in the presence of bacterial cell wall components, chitin induced potent pro-inflammatory immune responses via the same signalling pathway. Cross-activation of Fcγ-receptor with TLR2, TLR4 or NOD2 resulted in a synergistic pro-inflammatory immune response. Translating this immunomodulatory function of chitin into pathogenesis of fungal infections must take various influencing factors into account: on the one hand, chitin can dampen immune responses and control cytokine overproduction by the stimulation of the antiinflammatory IL-1Ra in the presence of immunoglobulins that are abundant in the serum. In addition, the strong anti-inflammatory signal facilitates the fungus to suppress the human defence mechanism and might even facilitate fungal invasion. On the other hand, although the exact fungal ligand is not known yet, TLR2, TLR4 and NOD2 are important for the antifungal host response since TLR2-/-, TLR4-/- and NOD2-/-mice were highly susceptible for invasive aspergillosis [8, 9] (Gresnigt *et al.*, submitted). This means that chitin can augment the immune system by amplification of TLR2-, TLR4- or NOD2-mediated signals activated either by fungal or bacterial pattern recognition receptor ligands. How this integrative concept of receptor-crosstalk changes during the

course of infection remains speculative, since littleis known about the cell wall structure and its composition change during germination and its interaction with host defense.

Although recognition is the first crucial step in successful antifungal host response, a strong T helper response is key for mediating effector functions to efficiently clear the fungus and establish an immunological memory. Pattern recognition receptors build the bridge by inducing the release of different innate cytokines that in turn activate the differentiation of naïve T cells towards specific T helper subsets. While invasive aspergillosis has been associated with a deficient Th1 and Th17 response, allergic aspergillosis is caused by increased and dysregulated Th2 responses. In **Chapter 4 and 5** we elucidated pattern recognition receptors, pathways and T helper subpopulations that are triggered-by *Aspergillus fumigatus*. *Aspergillus* can induce various specific T helper cytokines, but especially IL-22 has gained attention due to its protective role in invasive aspergillosis [10], in contrast to its detrimental role in allergic aspergillosis [11]. In **Chapter 4** we identified that the *Aspergillus*-specific IL-17 and IL-22 responses triggered by conidia and hyphae were dependent on TLR4, while blocking TLR2 increased these responses. IFNγ production was independent of TLR2 and TLR4. Interestingly, complement receptor 3 (CR3) was crucial for the initiation of the Th1 and Th17 subset, while the dectin-1 receptor was redundant. This was rather unanticipated, since dectin-1 is one of the main receptors in the recognition of *Candida albicans* and *Aspergillus* hyphae that abundantly express β-glucan on their surface [3]. By studying the cytokine co-expressing cells induced by *Aspergillus,* we identified that IL-22 was not restricted to a specific Th subset, but that these cells are able to co-express IL-17 as well as IFNγ. This indicates that *Aspergillus* does not induce one specific type of T helper response, but rather induces a diverse plastic T helper response. This T helper plasticity might contribute to a faster and more efficient defence of *Aspergillus*. The induction of IL-17 and IL-22 induced by *Aspergillus* conidia and hyphae was dependent on IL-1β and TNFα. This is an important finding given the widespread use of biologicals that target these cytokines. A few clinical reports have described the development of invasive aspergillosis after treatment with TNF blockers [12, 13]. This should raise the clinicians' awareness for a better fungal surveillance during treatment with biologicals that target cytokines that are important for the anti-*Aspergillus* host defence

In **Chapter 5** we investigated the pattern recognition pathways leading to Th2 responses induced by *Aspergillus*, and used this knowledge to discover potential novel corticosteroid-sparing therapeutic options. *Aspergillus* conidia were unique in triggering Th2 responses in human PBMCs, while no bacteria or fungi other than *Aspergillus*, or single fungal cell wall components, showed the capacity to induce IL-5 and IL-13. Interestingly, also *Aspergillus* hyphae failed to induce IL-5 or IL-13 despite their potent

IL-17- and IL-22-inducing capacity. We identified complement receptor 3 recognition and phagocytosis as key pathways involved in *Aspergillus*-specific Th2 responses. Surprisingly, none of the well-known PRRs such as TLR2/4 or dectin-1 were involved in the *Aspergillus*-specific Th2 response. This mechanism might prevent us from triggering an allergic response just by inhalation of fungal spores that loose their melanin and rodlets. ABPA patients show a unique *Aspergillus*-specific elevated Th2/Th1-ratio that could be restored by recombinant IFNγ, but not by other biologicals such as anti-TNFα or IL-1R antagonist. This is in line with some reports that describe an association of exaggeration of ABPA with TNFα blockers [14]. These data give a rationale to investigate IFNγ treatment in ABPA patients since it will supplement the *Aspergillus*-specific IFNγ deficiency on the one hand, and dampen the elevated *Aspergillus*-induced Th2 response on the other hand.

Several studies suggest that ABPA shares the same pathophysiologicalmechanisms as asthma. Interestingly, ABPA is more common in India compared to Europe, although asthma has a much higher prevalence in Europe compared to India. Furthermore, only a small percentage of people exposed to the high environmental fungal burden develop ABPA [15]. This might indicate a strong genetic factor influencing the susceptibility and pathogenesis of ABPA. Previous genetic studies have investigated candidate genes and confirmed important pathways of the already known antifungal host response, but this approach lacks the opportunity to find novel gene associations and has mainly been performed in patients with European descent. In **Chapter 6** we recruited patients with ABPA or asthma from India and performed a gene wide association study (GWAS). We used a systems biology approach to combine RNA expression analysis and functional genomics to investigate the pathogenesis of ABPA in India. Unexpectedly we did not find classical genes associated with the pathogenesis of asthma and allergy. Suggestive associations were found however in genes that interacted with the transcription factor STAT3, in genes that regulate the expression of type I interferons, and the vitamin D receptor. Functional validation confirmed the role of STAT3 in the *Aspergillus*-specific T cell response, especially during IL-17 differentiation. Further, we observed elevated IL-6 and IL-17 levels in the serum of ABPA patients, and ABPA serum had the capacity to skew the immune response of healthy PBMCs towards a pro-inflammatory profile. These findings are in contrast to earlier studies that have described ABPA as a classical Th2-disease, and suggest an important role of Th17 as well. Our data are reminiscent of a severe corticosteroids-resistant asthmatic phenotype, in which Th2/Th17 double-positive cells have been found in bronchoalveolar fluid [16] and blocking the STAT3 pathway had beneficial effects in airway inflammation in a multi-allergen challenged mouse model [17]. Taken together these findings suggest that the pathophysiology of ABPA might be

dependent on dysregulated Th17 responses in addition to an exaggerated Th2 response. How the modulation of these cytokines might be a beneficial adjuvant treatment option remains to be elucidated.

Our study could also confirm known pathophysiological mechanisms: vitamin D supplementation prevented the development of ABPA in CF patients that has been demonstrated in a clinical trial [18]. Therefore, modulation of type I interferons, STAT-3 inhibition or vitamin D supplementation might represent treatment options in ABPA which could prevent the use of conventional corticosteroid treatment regimen and might lead to a more personalized treatment.

The species *A. fumigatus* is the most common cause for invasive aspergillosis in patients with hemato-oncological malignancies, while *A. nidulans* is only a disease-causing organism in chronic granulomatous disease. [19]. This observation brought us to question how the host defence mechanisms between these two *Aspergillus* species differ. This might lead to an explanation why there is a higher prevalence of *A. nidulans* in an immune system lacking NADPH-oxidase activity. In **Chapter 7** we observed that *A. nidulans* had a lower capacity to induce reactive oxygen species in healthy immune cells, but could induce a significant higher pro-inflammatory response compared to *A. fumigatus* conidia. Moreover, *A. nidulans* was phagocytosed at a slower rate. Although we did not perform these experiments in NADPH-oxidase deficient settings, the different kinetics of innate immune recognition and phagocytosis might help us to explain why the epidemiological distribution differs so significantly. Hemato-oncological patients are deeply immunocompromised and are deficient in several protective immune mechanisms, such as phagocytosis and cytokine responses; thus they are susceptible to any *Aspergillus* species explaining why the species distribution of invasive aspergillosis follows the environmental distribution. In contrast, CGD patients have an isolated defective phagocytic machinery and ROS production in neutrophils and monocytes, while other immune mechanisms such as recognition, cytokine signalling pathways and T cell responses are not defective. CGD patients are exposed to the fungal pathogens for many years, which means that an accumulative exposure to *A. nidulans* in a defective phagocytic system can shift the distribution towards an increase to the less efficiently phagocytosed species, such as *A. nidulans*. Although the mechanism remains speculative our observations might contribute to explain the unique susceptibility of CGD patients to *A. nidulans* infections, and the higher mortality of *A. nidulans* infections. Further research needs to be performed to elucidate the exact reason why patients with CGD are susceptible to *A. nidulans.*

DEFICIENT T HELPER RESPONSES IN CLINICAL SETTINGS

In **Chapters 4** to **6** we demonstrate the importance of T helper responses for the *Apergillus*-specific host response with special emphasis on the recognition pathways that drive the immune response towards a certain Th response. The second part will focus on the role of T helper responses in different clinical settings. We selected patients with *Aspergillus* osteomyelitis of the skull base, patients with chronic mucocutaneous candidiasis and hyper IgE syndrome, as well as patients with other opportunistic infections such as non-tuberculous mycobacteria (NTM). NTMs play an important role in ABPA patients: like Aspergillus they are opportunistic pulmonary pathogens and chronic colonizers of the ABPA lung. Moreover, both are often present concomitantly.

Local *Aspergillus* infection, such as *Aspergillus* skull base osteomyelitis (SBO), is rare , especially in seemingly immunocompetent patients [20]. We studied the *Aspergillus*-specific T cell responses in six apparently immunocompetent patients with SBO due to *Aspergillus* in **Chapter 8** and observed a specific defect in their Th17 response. While the innate immune response did not differ between patients and healthy volunteers, the adaptive Th17 responses were significantly decreased after stimulation with fungal pathogens in the patients with SBO. This might help to explain why the seemingly immunocompetent patients were susceptible for the *Aspergillus* skull base infection, since Th17 plays a protective role in preventing invasive fungal disease. This observation could help to explore immunomodulatory treatment strategies next to the common antifungal treatment that would induce optimal Th17 responses in these patients.

The IL-17 deficiency associated with *Aspergillus* SBO was a new finding. In contrast, probably the best-studied patients with IL-17 deficiency are patients with chronic mucocutaneous candidiasis (CMC) and hyper IgE syndrome (HIES), both highly susceptible to fungal infections [21]. Less is known about their capability to differentiate towards T helper responses other than Th17. Interestingly, their predisposition to develop allergies differs tremendously: while allergies are only rarely reported in CMC patients, HIES patients can present with atopic eczema, eosinophilia and high IgE levels. In **Chapter 9** we investigated the cytokine profiles of allergic T helper (Th) 2 and 9 responses in CMC and HIES patients. Since IL-9 can be derived from the Th17 population, we also focused on IL-9 and explored the capacity of IL-9 induction by fungi in the setting of Th17 deficiency. We described the induction of Th2 and Th9 responses of four CMC and four HIES patients and the capability of these Th responses to be augmented by IL-4 to skew towards an allergic phenotype. CMC patients were completely deficient in allergic responses, namely IL-5, IL-13 and IL-9 production. In contrast, HIES patients were able to respond with a robust Th2 response. However their IL-9 production, especially in the presence of IL-4 co-stimulation was defective. Flowcytometric analysis revealed a

lower frequency of IL-9+ cells derived from the Th17 population, suggesting that the IL-9 deficiency is caused by a loss of the Th17 subset in HIES. These data provide evidence that the Th17 subset is important for optimal IL-9 responses induced by fungi. How these data can directly be translated to the clinical setting remains to be elucidated. Restoring IL-9 responses and thereby supporting the IL-17 response might be beneficial for the control of the fungal pathogen, a strategy that has been unexplored to date.

Our studies suggest that IL-17 and IL-22 are very important cytokines to control fungal pathogens at the site of the mucosa. But, what about other environmental bacteria that are facultative pathogens? Patients with cystic fibrosis that are defective in ciliary clearance and optimal innate immune responses are also often chronically colonized or infected with NTMs. Especially *M. abscessus* is very difficult to diagnose and to treat and moreover might be a reason why a CF patient is rejected for lung transplantation [22, 23]. In CF patients diagnosed with a chronic *M. abscessus* infection we observed a complete *M. abscessus*-specific IL-17 deficiency, in contrast to patients that were infected with other NTMs. This might be one of the reasons why these patients had a specific increased susceptibility to *M. abscessus.* In **Chapter 10** we deciphered the general and pathogen-specific immune cytokine profiles of two CF patients with chronic *M. abscessus* infection and ABPA. Compared to healthy controls, the CF patients showed a general exaggerated innate immune response. The *Aspergillus*-specific T helper response was skewed towards Th2, which is in line with their underlying ABPA. Notably, the *M. abscessus*-specific Th17 response of the CF patients was entirely deficient, despite their ability to produce IL-17 in response to *C. albicans*. These data are the first to report a possible important role for IL-17 in the human host defense against *M. abscessus* in patients with CF.

Patients with a unique body phenotype with scoliosis and pectus excavatus have been described to have a higher prevalence of non-tuberculous mycobacterial (NTM) infections. This association has been known for quite some time and is also called the Lady Windermere syndrome [24]. We performed whole exome sequencing in eleven patients with Lady Windermere Syndrome and their family members to identify a new molecular mechanism explaining the higher prevalence and susceptibility for NTM infections. In **Chapter 11**, we identified novel mutations in the *MST1R* gene in 4 out of the 11 patients. MST1R has been shown to be important for optimal bronchial cilia function. In addition, we demonstrate a second role for the MST1R in this syndrome, since patients carrying the mutation were deficient in IFNγ production, an important cytokine in the host defence against NTM infection. In further functional analysis, a specific defect in IFNγ production in response to NTM was observed when MST1R was blocked, while blocking MST1R did not alter other innate cytokines and IL-10. This suggests a dual role for MST1R in the

pathogenesis of Lady Windermere Syndrome by playing a role in optimal pulmonary cilia function, as well as anti-NTM host defence. It remains to be elucidated whether MST1R is involved in the IFN responses via its natural ligand MSP, or whether it is a direct PRR. Thus, we describe a genetic association with MST1R with the long known enigmatic Lady Windermere Syndrome, and provide novel functional insights into MST1R function in this disease that can be clinically relevant for adjunctive immunotherapy.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The field of fungal immunology is very dynamic and much knowledge has been gained due to the research that has been performed during the last decade. Since fungal infections are complex in many ways, there are still many challenges ahead of us. Diagnostic tools and treatment regimens are limited, antifungal drugs can come with severe side effects and high costs, and invasive fungal infections still have an unacceptable high rate of mortality. A thorough understanding of the molecular mechanisms underlying the anti-fungal host response is urgently needed to develop target-specific and immune-modulatory treatment strategies to support the conventional treatment in order to improve patient outcome.

A healthy immune system is able to control the inhaled *Aspergillus* conidia. We described the Fc-γ pathway as one of the underlying mechanisms recognizing the fungal cell wall polysaccharide chitin and playing a role in the initiation of an immune response against *Aspergillus*. The fact that patients with hypogammaglobulinemia suffer from recurrent respiratory infections emphasizes the importance of immunoglobulins in control and defence of pulmonary pathogens [25]. Chitin-binding antibodies can mediate immunomodulatory effects of chitin as described in this thesis. Vaccination with antigens and development of antigen-specific antibodies is one of the most resounding achievements in the prevention of infections. Up to date, vaccination against fungal infections was only successfully proven in mouse models, and no fungal vaccine is on the market for humans. Future research must be performed to decipher fungal antigens that are most protective against aspergillosis [26]. Chitin is certainly a promising vaccine candidate not only as an antigen itself, but also due to its immunomodulatory function; it has syngergistic effects with several pathogen associated molecular patterns such as LPS providing the rationale to study the effects of chitin together with immunogenic *Aspergillus* proteins in further studies.

Notably, patients with fungal infections are severly immunocompromised lacking neutrophils or antigen-specific T helper responses that most likely makes a vaccination inefficient. To overcome this problem, more studies need to be performed to elucidate,

how either active vaccination before the start of chemotherapy or hemato-oncological stem cell transplantation, or passive immunisation with fungal-specific antibodies during immunosuppression can help to compensate the deficiency. Moreover, adoptive transfer of *Aspergillus*-specific T cells has never been successfully performed in a patient with invasive aspergillosis, although experimental data look promising. Therapeutic or prophylactic adoptive transfer of *Aspergillus*-specific T cells from the stem cell donor to the recipient needs to be improved and should be part of future research.

Aspergillus has the capacity to induce a variable set of T helper responses, ranging from protective Th1 and Th17 responses to allergic Th2 responses. A balanced IL-1 response is crucial for a successful anti-*Aspergillus* host response [27] and especially for the differentiation towards adaptive immune responses that is a fine-tuned process influenced by IL-1. On the one hand, IL-1Ra is beneficial by dampening allergic responses, on the other hand IL-1Ra suppresses the beneficial Th1 response as well. Unexpectedly, we observed a possible Th17-driven pathogenesis of ABPA in functional genomic studies. This gives a rationale to explore the efficacy of anakinra (recombinant IL-1Ra) treatment in ABPA patients since IL-17 responses can be inhibited by anakinra. Moreover, patients with ABPA were shown to have also defective Th1 responses. The rationale to treat with IFNγ would have two sides: on the one hand it supplements the IFNγ deficiency and on the other hand it will dampen exaggerated Th2 responses. These two immunomodulatory strategies for ABPA are currently under investigation.

The field of immunodeficiencies is very broad and far from being entirely understood. Different approaches were used in this thesis to decipher some of the defective mechanisms in these patients. First, we wanted to decipher the pathological mechanism in patients with rare infections explaining their higher susceptibility to a specific pathogen. We observed a pathogen-specific IL-17 deficiency in patients with *Aspergillus* SBO and CF patients with *M. abscessus.* This points to a unique interaction of pathogen and immune cells and treatment should not be targeted only at eliminating the pathogen with antibiotics, but should also be focussed on restoring this defect. Adjuvant immunotherapy by augmenting a cytokine deficiency or blocking an exaggerated immune response remains a promising treatment option for the future, such as IFNγ or IL-1Ra. Secondly, we used immune cells isolated from patients with primary immunodeficiencies as an experimental setup. The natural IL-17-deficient setting as seen in CMC and HIES patients was used to decipher the allergic Th2 and Th9 responses. This strategy of using cells from patients with primary immunodeficiencies helps to investigate specific research hypotheses that arise from clinical observations and brings these findings back to the patients and even patients with diseases other than fungal infections.

Innovative diagnostic, prevention and immune-modulatory treatment options are strongly needed to overcome our limited possibilities in the management of fungal and other opportunistic infections.

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CHAPTER 13

Nederlandse samenvatting

List of publications

Curriculum vitae

Dankwoord

Nederlandse samenvatting en conclusie

Schimmels zijn eukaryote micro-organismen die overal in de natuur voorkomen, waar ze een waardevolle bijdrage leveren aan het ecosysteem door het afbreken van organische stoffen. Mensen worden permanent blootgesteld aan schimmels, maar gelukkig heeft een gezond afweersysteem verschillende mechanismen om infecties met schimmels te controleren. De gist *Candida* komt als commensaal voor op de menselijke mucosa en wordt getolereerd door het immuunsysteem in samenwerking met het microbioom. Sporen van de schimmel *Aspergillus* worden door de lucht verspreid en op een dagelijkse basis in grote aantallen geïnhaleerd, echter heeft de mens efficiënte mechanismen om deze sporen uit de long te elimineren.

In patiënten met een verstoord afweersysteem, kunnen deze schimmels pathogene capaciteiten ontwikkelen en ernstige infecties veroorzaken. Chemotherapie voor behandeling van kanker is een van de meest frequente oorzaken van verworven immunodeficiëntie. Daarnaast zijn verschilde genetische defecten geïdentificeerd die een verhoogde gevoeligheid voor schimmelinfecties kunnen veroorzaken. Deze genetische variaties variëren van polymorfismen die de kans op schimmelinfecties vergroten in een immuun gecompromitteerde context, tot monogenetische mutaties die geassocieerd zijn met een aangeboren immunodeficiëntie.

In tegenstelling, een hyperreactief immuunsysteem kan ook bijdragen aan ontwikkeling van allergische infecties. *Aspergillus* heeft potente allergenen die een hyperreactief immuunsysteem kunnen triggeren en bijdragen aan ontwikkeling van ernstig astma met schimmelsensitisatie. De allergische reactie kan in ernstige gevallen leiden tot allergische bronchopulmonale aspergillosis, in het bijzonder in patiënten met ernstig astma of cystische fibrose.

Therapeutische mogelijkheden voor behandeling van zowel invasieve en allergische schimmelinfecties zijn gelimiteerd. Immuun-modulerende therapieën worden gezien als een aantrekkelijke strategie om de behandeling van schimmelinfecties te verbeteren. Echter, voor de ontwikkeling van deze nieuwe immuun-modulerende therapieën is het van cruciaal belang dat we de moleculaire mechanismen van schimmelinfecties beter begrijpen.

HERKENNING VAN *ASPERGILLUS* EN INDUCTIE VAN DE T-HELPERRESPONS

Bij het bestuderen van de interactie tussen gastheer en schimmels moet men altijd twee kanten in overweging nemen: (1) hoe de schimmel probeert te overleven in de gastheer, en (2) hoe de gastheer de schimmel onder controle houdt om infectie te voorkomen.

Bij invasieve schimmelgroei ondergaat de schimmel een verandering van morfologie, namelijk het uitgroeien tot een filamenteuze vorm genoemd hyphae waarmee de schimmel het lichaam kan indringen en een infectie kan veroorzaken. Om verschillende morfologische vormen te kunnen herkennen heeft het afweersysteem verschillende pattern regocognition receptors (PRRs) om een immuunrespons op te wekken (1). In **hoofstuk 2** geven wij een overzicht van die meest bekende PRRs, die betrokken zijn bij de herkenning van de twee meest voorkomende pathogene schimmels, namelijk *Candida* en *Aspergillus*. Slechts een klein aantal van PRRs en de daarmee verbonden signaalcascaden betrokken bij de afweer tegen schimmels zijn bekend. De interactie en crosstalk tussen verschillende signaalmoleculen is de reden, waarom het afweersysteem een specifieke aangeboren en adaptieve immuun respons kan opwekken (2). Het synergisme tussen dectin-1 en TLR2, de activatie van de dectin-1 gemedieerde fagocytose en activatie van het NLRP3 inflammasoom zijn de best beschreven mechanismen hoe de immuunrespons tegen schimmels gepolariseerd wordt (3, 4). Er is een complexe interactie tussen de verschillende immuuncellen. Neutrofielen, monocyten, macrofagen, NK-cellen, T-cellen, innate lymphoid cellen, epitheelbarrières en endotheel werken samen voor een effectieve immuunrespons tegen schimmels. Polysachariden van de celwand van de schimmel zijn liganden voor de PRRs. Verschillende studies hebben onderzocht welke receptoren de verschillende schimmel polysachariden kunnen herkennen, met als doel de moleculaire mechanismen van de schimmelherkenning beter te begrijpen en betere therapeutische strategieën te kunnen ontwikkelen. In **hoofdstuk 3** hebben we de immunomodulatoire capaciteit van chitin onderzocht. Chitin is een moelucuul van de celwand van *Aspergillus*. Verschilde aspecten van de capaciteit van chitin om het immuun systeem te activeren zijn reeds beschreven. Bueter *et al.* hebben beschreven dat chitin een partikel is zonder immunologische functie, maar Wagener *et al*. hebben gevonden, dat chitin een sterke anti-inflammatoire capaciteit bezit, wat gemedieerd wordt door mannosereceptor, TLR9 en NOD2 (5, 6). Een interessante gedachte is dat verschillen in de experimentele setup in samenhang met zuiverheid, grote, vorm en ook de isolatie van chitinpartikels een verklaring kunnen zijn voor de conflicterende resultaten met betrekking tot de immunogeniciteit van chitin tussen verschillende publicaties (7). In onze studie hebben we chitinpartikels van *Aspergillus* met een partikelgrootte van < 0,5 µm gebruikt. Deze partikels hebben een hoge graad aan acetylatie en zijn

kortdurend gesonificeerd middels ultrasonicatie. Mogelijke contaminatie van β-glucan is verwijderd door degradatie met het enzym β-glucanase. Middels stimulatie van PBMCs met deze partikels in de aanwezigheid van humaan serum hebben we de Fcγ/Syk/PI3K cascade geïdentificeerd, welke geïnduceerd wordt door chitin, om een sterke anti- en pro-inflammatoire immuunrespons te induceren. Ten eerste, hebben we gevonden dat *Aspergillus*-chitin een sterke activator van een anti-inflammatoire immuunrespons is, gekarakteriseerd door productie van de anti-inflammatoire cytokine IL-1Ra, maar geen pro-inflammatoire cytokines. Immuunglobulines in het humane serum zijn belangrijke opsonines, om de Fcγ/Syk/PI3K cascade te activeren. Wij hebben gevonden dat deze cascade cruciaal is voor de chitinspecifieke IL-1Ra-inductie.

Ten tweede hebben we laten zien dat chitin in aanwezigheid van andere immunologisch actieve bacteriële celwandcomponenten een potente pro-inflammatoire immuunrespons kan induceren. Hierbij maakt chitin gebruik van dezelfde cascade, echter door een samenwerking tussen de Fcγ-receptor met TLR2, TLR4 of NOD2 wordt er een synergistische pro-inflammatoire immuunrespons geïnduceerd. Samengenomen, weten we nu verschillende mechanismen waarmee chitin zijn immuunmodulerende functie uitoefent. Chitin kan, in de aanwezigheid van immunoglobulinen, de immuunrespons dempen door IL-1Ra te induceren. Als gevolg van dit sterke anti-inflammatoire signaal, is het mogelijk voor de schimmel om de humane immuunrespons te onderdrukken en daardoor mogelijk schimmelinvasie te faciliteren. Ondanks dat de exacte schimmelliganden voor TLR2, TLR4 en NOD2 nog onbekend zijn, is er voor deze receptoren aangetoond dat ze een belangrijke rol spelen in de inductie van de immuunrespons tegen schimmels (8, 9) (Gresnigt *et al.*, submitted). In tegenstelling tot de anti-inflammatoire rol van chitin zou dit kunnen betekenen dat chitin bijdraagt aan een adequate inductie van de antifungale immuunrespons door TLR2-, TLR4- of NOD2-gemedieerde signalen te amplificeren.

Hoe de crosstalk tussen receptoren verandert gedurende het verloop van infectie blijft speculatief, omdat niet veel bekend is over hoe de *Aspergillus*-celwand verandert tijdens de groei van de schimmel en hoe dit een interactie met de immuunrespons heeft. Herkenning van schimmelmoleculen is de eerste cruciale stap van een succesvolle afweerreactie tegen de schimmel. Daarnaast is een sterke T-helperrespons de sleutel voor inductie en versterking van immunologische effectormechanismen die op een efficiënte manier de schimmel opruimen en een immunologisch geheugen generen. Pattern recognition receptoren slaan de brug tussen het aangeboren immuunsysteem en de adaptieve immuunrespons. Dit doordat deze receptoren de uitscheiding van cytokines in cellen van het aangeboren immuunsysteem induceren, deze cytokines induceren op hun beurt naïeve T-helpercellen die vervolgens differentiëren om een specifieke

T-helperrespons genereren. Aan de ene kant is invasieve aspergillosis geassocieerd met een deficiënte van de Th1- en Th17-respons, anderzijds wordt allergische aspergillosis veroorzaakt door een verhoogde en foutief gereguleerde Th2-respons.

In **hoofdstukken 4 en 5** hebben we onderzocht welke pattern recognition receptoren, signaaltransductie en T-helpersubpopulaties door *Aspergillus fumigatus* geactiveerd worden. *Aspergillus* kan verschillende specifieke T-helpercytokines induceren, maar vooral de inductie van IL-22 wordt belangrijk geacht door de protectieve rol in invasieve aspergillosis (10), in tegenstelling tot een destructieve rol in allergische aspergillose (11). In **hoofdstuk 4** hebben we geïdentificeerd welke receptoren de *Aspergillus*-specifieke IL-17- en IL-22-respons induceren. We hebben kunnen laten zien dat deze respons afhankelijk is van TLR4, maar in tegenstelling het blokkeren van TLR2 resulteerde in een versterking van deze T-helperrespons. Enigszins verrassend was dat complement receptor 3 (CR3) cruciaal was voor de inductie van een Th1- en Th17-respons, maar dat de dectin-1 receptor overbodig bleek te zijn. Dit was een onverwachte bevinding, omdat dectin-1 geïdentificeerd is als een van de belangrijkste receptoren voor de herkenning van *Candida albicans* en *Aspergillus* hyphae (3). Verder hebben we de co-expressie van verschillende cytokines in *Aspergillus*-geïnduceerde T-celpopulaties bestudeerd, en vonden dat IL-22 niet door een specifieke T-helpersubklasse geproduceerd wordt, maar dat er een grote plasticiteit is in het cytokinerepertoire van *Aspergillus*-geïnduceerde T-celpopulaties. Dit betekent dat *Aspergillus* niet de specifieke T-helperpopulaties zoals Th1 en Th17 induceert, maar eerder een diverse plastische T-helperrespons. Deze T-helperplasticiteit zou kunnen bijdragen bij een snellere een efficiëntere immuun respons tegen *Aspergillus*. Die inductie van IL-17 en IL-22 door *Aspergillus* conidia en hyphae is sterk afhankelijk van de cytokines IL-1β en TNFα. Dat belangrijke informatie met de blik op het wijdverspreid gebruik van biologicals voor de behandeling van inflammatoire ziekten die specifiek deze cytokineresponsen beïnvloeden. Verschillende casus beschrijven de ontwikkeling van een invasieve aspergillosis na behandeling met TNFα blokkers (12, 13). De associatie tussen verschillende cytokinecascades en een effectieve inductie van de afweerreactie tegen *Aspergillus*, is een belangrijk aspect dat belangrijk is voor de kliniek. Bijvoorbeeld patiënten die met specifieke biologicals behandeld worden, zouden intensief gemonitord moeten worden voor de ontwikkeling van schimmelinfecties.

In **hoofdstuk 5** hebben we de PRRs geanalyseerd die betrokken zijn bij de inductie van een Th2-respons door *Aspergillus*. Kennis met betrekking tot de moleculaire mechanismen van de Th2-respons kan gebruikt worden, om nieuwe gerichte therapeutische opties te ontwikkelen die de Th2-respons kunnen remmen. Dit zou een aanlokkelijke strategie zijn om de huidige veel bredere immunosuppressieve therapie in de vorm van corticosteroïden te vervangen. *Aspergillus* conidia zijn uniek in hun potentie om een

Th2-respons in humane PBMCs te induceren. Andere bacteriën, schimmels, of zelfs opgezuiverde celwandcomponenten van de schimmel hadden niet de capaciteit om een IL-5 of IL-13 respons te induceren. Daarnaast is het heel interessant dat wij vonden dat de hyphae van *Aspergillus* ook geen IL-5 of IL-13 kunnen induceren, ook als zij wel in staat een sterke IL-17 en IL-22 respons te induceren. Deze observatie ligt in de lijn der verwachting, namelijk specifiek de inhalatie van schimmel sporen wordt gezien als de belangrijkste trigger van een astmatische respons in ABPA. We hebben geïdentificeerd dat complement receptor 3 en fagocytose van de sporen cruciaal is voor de inductie van de *Aspergillus*-specifieke Th2-respons. Onverwacht was dat geen van de PRRs die betrokken zijn bij een efficiënte afweer tegen invasieve aspergillosis (zoals bijvoorbeeld TLR2/4 of dectin-1), een rol speelden in de inductie van de *Aspergillus*-specifieke Th2-respons. Het feit dat deze receptoren niet betrokken zijn bij de inductie van de Th2-respons zou een beschermend mechanisme kunnen zijn tegen allergische reacties geïnduceerd door de schimmel. Wanneer de T-celrespons van gezonde individuen en ABPA-patiënten werd vergeleken, observeerden wij dat ABPA-patiënten een unieke verhoging hebben van de ratio tussen de *Aspergillus*-specifieke Th2- en Th1-respons. Mogelijk zou deze verstoring van de Th1/Th2 balans een belangrijke rol kunnen spelen in de pathogenese van ABPA en astma met schimmelsensitisatie. Daarom hebben we getracht deze balans te herstellen met behulp van biologicals die gericht zijn op de immuunrespons. Wij vonden dat *in vitro* toepassing van biologicals zoals anti-TNFα- of IL-1R-antagonisten deze balans niet konden herstellen, echter recombinant IFNγ herstelde de disbalans tussen Th2 en Th1. Reeds eerder zijn exacerbaties van ABPA door het gebruik van TNF-antagonisten beschreven (14). Dit in samenhang met onze data pleit voor klinische studies die onderzoeken of het geven van IFNγ-therapie in ABPA-patiënten potentie heeft om ten eerste een *Aspergillus*-specifieke IFNγ-deficiëntie te compenseren, en ten tweede en verhoogde *Aspergillus*-geïnduceerde Th2-respons kan verlagen, en daarmee het allergische fenotype terug te dringen en exacerbaties te voorkomen.

Verschillende studies suggereren dat ABPA en astma een vergelijkbare onderliggende pathogenese hebben. Echter komt ABPA in India duidelijk vaker voor in vergelijking met Europa, terwijl de prevalentie van astma veel hoger is in Europa. Slechts een gering aantal mensen, dat dagelijks in contact komt met grote hoeveelheden schimmelsporen, ontwikkelt hierdoor ABPA (15). Dat duidt op een sterke genetisch factor die gevoeligheid voor ABPA beïnvloedt. Eerdere genetische studies hebben genetische kandidaten bestudeerd en konden zo van verschillende belangrijke signaalcascades, die al bekend waren in de immuunrespons tegen *Aspergillus,* bevestigen dat ze ook een rol spelen bij de pathogenese van ABPA. Deze benadering heeft echter niet geleid tot identificatie

van nieuwe genetische associaties. Daarnaast waren deze eerdere studies uitgevoerd met een cohort met een Europese genetische achtergrond. In **hoofdstuk 6** hebben wij ABPA-patiënten uit India gerekruteerd en met behulp van een immunochip assay gegenotypeerd. We hebben gebruik gemaakt van systeembiologie, expressie-analyse en functionele genetische analyse om de pathogenese van ABPA in de Indische populatie te bestuderen. Onverwacht was dat we geen typisch genetisch patroon hebben kunnen identificeren dat geassocieerd was met de pathogenese van astma en allergie. Wanneer wij de ABPA-patiënten met de controlegroep vergeleken, hebben wij significante associaties met genen gevonden, betrokken bij de transcriptiefactor STAT3, genen die de expressie van *IRF8* reguleren, en een gen, dat de vitamine D-receptorexpressie reguleert. Met behulp van functionele validatiestudies hebben we de rol van STAT3 in de *Aspergillus*-specifieke T-celrespons kunnen bevestigen, in het bijzonder in de Th17-differentiatie. Verder hebben wij gezien dat naast een verhoogde Th2-respons ook IL-6 en IL-17 verhoogd zijn in het serum van ABPA-patiënten. Daarnaast hebben we gevonden dat ABPA-serum de capaciteit heeft om de immuunrespons van PBMCs geïsoleerd uit gezonde individuen naar een pro-inflammatoir profiel te brengen. Deze bevindingen staan in tegenstelling tot vroegere studies die ABPA als een typische Th2-gerelateerde ziekte beschrijven. In de bronchoalvelolaire lavage van ABPA-patiënten zijn Th2/Th17-dubbelpositieve celen gevonden (16), en het blokkeren van het STAT3-signaal heeft een positief effect op de luchtweginflammatie in allergiemuismodellen (17). Samenvattend suggereren deze resultaten dat the pathofysiologie van ABPA afhankelijk is van een slecht gereguleerde pro-inflammatoire Th1- respons in samenhang met een verhoogde Th2-respons. Verder zijn type I interferonen geassocieerd met chronisch mucocutane candidiasis en betrokken bij de pathogenese van ABPA (18). Hoe de modulatie van cytokines kan bijdragen als een aanvullende therapie moet nog nader onderzocht worden in toekomstige studies. Onze studie heeft verschillende bekende pathologische mechanismen kunnen bevestigen. Een klinische trial heeft aangetoond, dat vitamine D-suppletie een beschermend effect heeft op de ontwikkeling van ABPA in CF-patiënten (19). De modulatie van type I interferonen, STAT3-inhibitie of vitamine D-suppletie zouden mogelijke nieuwe therapeutische opties zijn voor ABPA.

Die species *A. fumigatus* is de meest voorkomende *Aspergillus*-soort, die invasieve aspergillosis in patiënten met haemato-oncologische onderliggende ziekten veroorzaakt. In tegenstelling, *A. nidulans* veroorzaakt zelden infecties in deze patiëntengroep en veroorzaakt alleen infecties in patiënten met chronische granulomateuze ziekte (CGD) (20). In **hoofdstuk 7** hebben wij gezien dat *A. nidulans* een lagere capaciteit heeft om reactieve zuurstofmoleculen te induceren in immuuncellen van gezonde individuen, echter induceert *A. nidulans* significant meer pro-inflammatoire cytokines in vergelijking

met *A. fumigatus*. Verder hebben we geobserveerd dat *A. nidulans* minder efficiënt gefagocyteerd wordt dan *A. fumigatus*. Ondanks dat deze experimenten niet in NADPH-oxidase deficiënte cellen zijn uitgevoerd, de verschillen in de kinetiek van aangeboren immuunherkenning en fagocytose kan mogelijk de verschillende epidemiologische verdeling te verklaren zijn. Hemato-oncologische patiënten hebben een sterk verzwakt afweersysteem en zijn deficiënt voor verschillen immuunfuncties, zoals fagocytose en het induceren van een cytokinerespons; dus zijn deze patiënten vatbaar voor *Aspergillus*-species. Dit verklaart mogelijk ook waarom de prevalentie van verschillende geïsoleerde *Aspergillus*-species in deze patiënten de verdeling in de omgeving volgt. In tegenstelling, CGD-patiënten hebben een geïsoleerd fagocytosedefect en deficiënte ROS-productie in neutrofielen en monocyten. Echter zijn andere immuunfuncties zoals pathogeenherkenning, cytokineproductie en T-celresponsen intact. CGD-patiënten zijn gedurende hun hele leven aan schimmels blootgesteld, wat betekend dat ze een cumulatieve blootstelling hebben aan *A. nidulans.* In patiënten met een levenslange gevoeligheid voor aspergillosis zal de verdeling verschuiven richting meer *A. nidulans* infecties, gezien de minder efficiënte fagocytose van deze schimmel. Onze observatie zou kunnen bijdragen aan de verklaring waarom GCD-patiënten een unieke vaatbarheid voor *A. Nidulans*-infecties hebben en zou daarnaast kunnen verklaren waarom infecties met *A. nidulans* geassocieerd zijn met een hogere mortaliteit. Er is zeker nog meer onderzoek nodig om dee exacte reden te vinden, waarom CGD patiënten zo gevoelig voor *A. nidulans* infecties zijn.

DEFICIËNTE T-HELPERRESPONS IN KLINISCHE SETTING

In **hoofdstukken 4 tot 6** hebben we gedemonstreerd dat de T-helperrespons belangrijk is voor de *Aspergillus*-specifieke immuunrespons. Het eerste deel van dit werk is gefocusseerd op het ontrafelen van liganden, receptoren en cytokines die betrokken zijn in de *Aspergillus*-specifieke immuunrespons in *in vitro*-modellen; daarentegen hebben we in het tweede deel de focus gelegd op de rol van T-helperresponsen in een klinische setting. We hebben verschillende patiënten onderzocht, zoals onder andere patiënten met lokale *Aspergillus*-infecties in de schedelbasis, patiënten met chronische mucocutane candidiasis en hyper-IgE-syndroom, maar ook patiënten met andere opportunistische infecties, zoals patiënten met infecties met niet-tuberculoze mycobacteriën (NTM). Opmerkelijk is dat de NTM-infecties veel overeenkomst delen met schimmelinfecties: beide zijn opportunistisch, veroorzaken infecties in de long en ABPA-patiënten zijn chronisch gekoloniseerd met *Aspergillus* en NTMs.

Lokale *Aspergillus*-infecties, zoals *Aspergillus*-schedebasisosteomyelitis (SBO) zijn uiterst zeldzaam (21). In **hoofdstuk 8** hebben wij de *Aspergillus*-specifieke T-celrespons in schijnbaar immuuncompetente patiënten met *Aspergillus*-SBO bestudeerd. Hierbij hebben we een specifiek defect in de Th17 respons gevonden. De stimulatie van cellen van zes patiënten met *Aspergillus*-SBO met bacteriële en schimmelantigenen heeft aangetoond dat de aangeboren immuunrespons tussen patiënten en controles niet verschillend is. Echter hebben deze patiënten wel een significant verlaagde Th17-respons na stimulatie met schimmelantigenen. Dit zou kunnen verklaren, waarom schijnbaar immuuncompetente patiënten gevoelig zijn voor *Aspergillus*-SBO, omdat de Th17-respons een belangrijke rol speelt in de verdediging tegen invasieve schimmelinfecties. Onze observatie zou kunnen helpen bij de ontwikkeling van een immuunmodulerende therapeutische strategie die gericht is op het induceren van een optimale Th17-respons.

De IL-17-deficiëntie geassocieerd met *Aspergillus*-SBO was een onverwachte bevinding. In tegenstelling, is IL-17-deficiëntie een van de best bestudeerde mechanismen in patiënten met chronische mucocutane candidiasis (CMC) en hyper-IgE-syndroom (HIES). Beide patiëntengroepen zijn extreem gevoelig voor het ontwikkelen van schimmelinfecties (22). Er is echter minder bekend over de andere T-helperresponsen die naast de Th17-respons ook een belangrijke rol in de gevoeligheid voor schimmelinfecties zou kunnen spelen. De predispositie om een allergie te ontwikkelen verschilt enorm tussen deze twee patiëntengroepen: allergieën zijn bijna nooit in CMC-patiënten gerapporteerd, maar HIES-patiënten zijn bekend met presentatie van atopisch eczeem, eosinofilie en hoge IgE-levels. In **hoofdstuk 9** hebben we de cytokineprofielen van Th2- en Th9-responsen in CMC- en HIES-patiënten bestudeerd. Omdat IL-9 geproduceerd kan worden door Th17-cellen, hebben we specifiek op IL-9 gefocust, en hebben onderzocht of CMC- en HIES-patiënten met een IL-17 deficiëntie wel IL-9 kunnen produceren.

We hebben de inductie van Th2- en Th9-responsen van vier CMC- en vier HIES-patiënten onderzocht. Om de capaciteit van deze cellen om specifiek een allergisch fenotype te induceren beter te kunnen beoordelen hebben wij IL-4 toegevoegd aan het kweekmedium. CMC-patiënten waren compleet deficiënt in het induceren van Th-responsen die geassocieerd zijn met allergie, namelijk IL-5, IL-13 en IL-9 met en zonder IL-4-toevoeging. In tegenstelling, HIES-patiënten hadden wel de capaciteit om een robuuste Th2-respons te induceren. Echter was de IL-9-productie in beide groepen deficiënt. Met behulp van flowcytometrie hebben we aangetoond dat IL-9+-cellen in de Th17-populatie te vinden zijn en HIES-patiënten een kleinere populatie van IL-9+-IL-17+-cellen hebben. Dat betekent dat de Th17-populatie ook belangrijk is voor de inductie van een optimale IL-9 respons. Hoe deze data direct naar een klinische setting vertaald kunnen worden, moet nog nader onderzocht worden. Het herstellen van een sterke

IL-9-respons en daarbij ook de Th17-populatie zou nuttig kunnen zijn voor een betere controle van schimmelinfecties.

Onze studies suggereren dat IL-17 en IL-22 belangrijke cytokines zijn in de afweer tegen schimmels op de mucosa. Patiënten met cystische fibrose (CF) hebben een deficiënte ciliaire clearance en zijn vaak chronisch gekoloniseerd of geïnfecteerd met NTMs. Vooral *M. abscessus* is een moeilijk te diagnosticeren en te behandelen mycobacterium en vaak een reden waarom patiënten afgewezen worden voor longtransplantatie (23, 24). We hebben in CF-patiënten die een chronische *M. Abscessus*-infectie hebben, een *M. abscessus*-specifieke IL-17-deficiëntie gevonden, in tegenstelling tot patiënten met andere NTM-infecties. Dit zou mogelijk een reden kunnen zijn waarom deze patiënten een verhoogde gevoeligheid voor *M. abscessus* hadden. In **hoofdstuk 10** hebben we het algemene en pathogeen-specifieke cytokineprofiel van twee CF-patiënten met chronische *M. Abscessus*-infectie en ABPA onderzocht. In vergelijking met de gezonde controles hebben de CF-patiënten een verhoogde aangeboren immuunrespons. De *Aspergillus*-specifieke T-helperrespons was richting Th2 gepolariseerd, wat past bij hun ABPA-fenotype. Wij vonden echter dat de *M. abscessus*-specifieke Th17-respons compleet deficiënt was, desondanks dat deze individuen wel de capaciteit hadden om IL-17 te produceren in respons op *Candida*. De *M. abscessus*-specifieke IL-17-defichientie kan mogelijk een rol spelen in de gevoeligheid van CF=patiënten om chronisch gekoloniseerd te zijn met *M. Abscessus*.

Een hogere prevalentie van NTM-infecties is ook beschreven bij patiënten met een unieke lichaameigenschappen zoals scoliose en *pectus excavatus*. Deze associatie is al lang bekend en word ook het Lady Windermere Syndrome genoemd (25). We hebben een 'whole exome sequencing' studie in 11 patiënten met Lady Windermere syndrome en hun familieleden gedaan om mogelijke onderliggende genetische defecten te identificeren die de hogere prevalentie en gevoeligheid voor NTM-infecties in deze patiëntengroep zou kunnen verklaren. In **hoofdstuk 11** beschrijven we een mutatie in het MSTR gen dat werd gevonden in 4 van de 11 patiënten. Het *MSTR1* gene is belangrijk voor een optimale bronchiale ciliare functie. Daarnaast hebben we een tweede rol voor MSTR1 in dit syndroom aangetoond, de patiënten met deze mutatie lieten een specifieke deficiënte IFNγ-productie zien. Verdere functionele analyse heeft aangetoond dat IFNγ-productie defect was na stimulatie met NTM in de aanwezigheid van een MST1-receptorblokker. Echter andere cytokines geproduceerd door het aangeboren immuunsysteem werden niet door deze blokkade beïnvloed. Deze bevinding suggereert een tweeledige rol voor MST1R in de pathogenese van Lady Windermere syndroom: ten eerste, de optimale controle van de pulmonale ciliaire functie en ten tweede een belangrijke rol in de immuunrespons tegen NTMs.

ALGEMENE CONCLUSIES EN TOEKOMSTPERSPECTIEVEN

Het veld dat de immunologie in schimmelinfecties onderzoekt is zeer dynamisch en er is veel nieuwe kennis opgedaan in de laatste jaren. Omdat schimmelinfecties zeer complex zijn, liggen er nog vele aantal uitdagingen voor ons. Diagnostische mogelijkheden en therapeutische strategieën zijn gelimiteerd, antimykotische medicatie heeft serieuze bijwerkingen en is duur, en invasieve schimmelinfecties hebben steeds nog een zeer hoge mortaliteit. Een breed inzicht in die moleculaire mechanismen van de immuunrespons tegen schimmels is dringend nodig om doelgerichte immuun-modulerende therapeutische strategieën te ontwikkelen. Deze nieuwe therapeutische strategieën zijn nodig om de conventionele therapie te ondersteunen en mortaliteit van deze infecties terug te brengen. Een gezond immuunsysteem kan ie geïnhaleerde *Aspergillus*-sporen gemakkelijk controleren. We hebben beschreven dat de Fc-γreceptor *Aspergillus*-chitin herkent en de immuunrespons tegen *Aspergillus* initieert. Het feit dat patiënten met hypogammaglobulinemia recidiveerde respiratorische infecties hebben, ondersteunt het belang van immunoglobulinen in de afweer tegen pulmonale pathogenen [25]. Het immuunmodulerende effect van chitin was gemedieerd door chitinbindende antilichamen. Vaccinatie met antigenen en ontwikkeling van specifieke antilichamen is een van de meest geweldig ontwikkelingen in preventie van infecties. Vaccinaties tegen schimmels zijn tot nu toe alleen nog in muismodellen succesvol getest. Toekomstig onderzoek dient de meest protectieve antigenen tegen *Aspergillus* identificeren [26]. Chitin is met zekerheid een veelbelovend vaccinatiekandidaat niet alleen als antigen zelf, maar het zou ook door zijn immuunmodulerende functie als adjuvant kunnen dienen. Bij de ontwikkeling van vaccinatiestrategieën moeten we niet vergeten dat patiënten met schimmelinfecties vaak immuuncomprimeerd zijn en geen neutrofielen of antigen-specifieke T-helperresponsen kunnen opwekken. Dit maakt een vaccinatie heel moeilijk en inefficiënt. Om dit probleem te overkomen zouden toekomstige studies zich er op kunnen richten om uit te zoeken, of een actieve vaccinatie voor een chemotherapie of stamceltransplantatie, of passieve vaccinatie met schimmelspecifieke antilichamen tijdens immuunsuppressie een voordelig effect kunnen hebben op het terugdringen van de incidentie en mortaliteit van schimmelinfecties in deze patiënten. Adoptieve transfer van *Aspergillus*-specifieke T-cellen is nooit succesvol in een patiënt met invasieve aspergillosis uitgevoerd, terwijl de experimentele data er veelbelovend uitzien. Therapeutische en profylactische adoptieve transfer van *Aspergillus*-specifieke T-cellen van donor naar zijn ontvanger moet nog sterk verbeterd worden. *Aspergillus* heeft de capaciteit een breed scala aan van T-helperresponsen te induceren. Dit reikt van de protectieve Th1respons tot de Th2-respons die betrokken is bij ontwikkeling van allergie. Een gebalanceerde IL-1-respons is cruciaal voor een succesvolle afweer tegen

Aspergillus [27] en in het bijzonder voor de differentiatie en polarisatie van de adaptieve immuunrespons. Ten eerste, IL-1Ra is een goede kandidaat voor het dempen van de allergische immuunrespons die een rol speelt bij ABPA, echter ten tweede kan IL-1Ra ook die nuttige Th1-respons onderdrukken. Enigszins onverwacht hebben we de observatie gemaakt dat ABPA-patiënten ook verhoogde IL-17-respons hebben, een sterke pro-inflammatoire respons die zou kunnen bijdragen aan de pathogenese van ABPA. Deze observatie zou een goede rationale zijn om de effectiviteit van Anakinra (recombinant IL-1Ra) therapie in ABPA-patiënten te onderzoeken, omdat de mogelijk schadelijke IL-17-respons door Anakinra sterk geremd kan worden.

Het veld van immuundeficiënties is zeer breed en veel immuundeficiënties zijn nog slecht bestudeerd. In dit proefschrift zijn verschillende strategieën toegepast om enkele immuundeficiënties verder te ontrafelen. Onze intentie was om de pathologische mechanismen in patiënten met zeldzame infecties te onderzoeken, vooral met het doel te verklaren waarom deze patiënten gevoelig zijn voor specifieke zeldzame pathogene. We hebben pathogeen-specifieke IL-17-deficiënties gevonden in patiënten met *Aspergillus*-SBO en CF-patiënten met een *M. Abscessus*-infectie. Dit wijst op een unieke interactie tussen pathogeen en het immuunsysteem die resulteert in pathogeen-specifieke immuun responsen. De therapie bij deze immuundeficiënties moet niet alleen op de eliminatie van het pathogeen door antimicrobiële medicatie focussen, maar vooral op de compensatie van de onderliggende cytokinedeficiëntie. Als experimentele setup hebben we primaire immuuncellen geïsoleerd van immuundeficiënte patiënten. De IL-17-deficiëntie zoals gezien in patiënten met CMC en HIES hebben we gebruikt, om de allergische Th2- en Th9-respons te onderzoeken. Deze strategie kan bijdragen om specifieke onderzoek hypothesen te bevestigen en zal het bijdrage aan nieuwe kennis die direct vertaald kan worden naar de behandeling van de patiënt.

Innovatieve diagnostische, preventieve en immuunmodulerende therapeutische opties zijn dringend nodig, om de gelimiteerde mogelijkheden van de behandeling van schimmelinfecties en andere opportunistische infecties te overkomen.

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˙ Contributed equally.

Curriculum vitae

Katharina Gößling (nee Becker) was born in Heidelberg, Germany on September 8th, 1986. She attended high school at the Gymnasium Fridericianum in Erlangen, Germany and received her diploma (Abitur) in 2005 with the major subjects mathematics and ancient Greek. From 2005 to 2012 she studied medicine at the University Hospital Hamburg-Eppendorf with internships in Zurich, Switzerland and Kumasi, Ghana. In 2012 she graduated in medicine with the main subject in Medical Microbiology and received the Approbation. During her study time she had chosen additional courses in molecular medicine with special focus on practical laboratory work.

Next to her medical studies in 2008 she started to work on her M.D. thesis at the Bernhard-Nocht-Institute for Tropical Medicine in Hamburg in the group of Prof. B. Fleischer under supervision of Prof. M. Jacobsen. She studied the immune response against *Mycobacterium ulcerans* in patients with Buruli ulcer. In 2012 she received the medical doctor degree.

In 2011 she performed an internship at the Department of Internal Medicine, Radboudumc, Nijmegen, under supervision of Dr. M. S. Gresnigt. She became interested in the immunology of fungal infections and started her Ph.D. research in the same department under the mentorship of Dr. F. L. van de Veerdonk, Prof. dr. L.A.B. Joosten and Prof. dr. M.G. Netea in 2012. During her Ph.D. project, Katharina presented her results at numerous national and international scientific conferences. She received the ECMM Young Investigator Travel Award for oral presentation at the TIMM 2013 in Copenhagen, Denmark, the Poster Award at the Gordon Research Seminar of Fungal Immunology 2015 in Galveston, Texas and the Oral Presentation Award at the 21st RIMLS PhD-retreat 2015. For research collaboration, she visited and worked in the laboratory of Prof. dr. A. Chowdhary at the V.P. Chest Institute in Delhi, India. She went to the Institute Pasteur in Paris to the Unité des *Aspergillus* of Prof. dr. J.-P. Latgé to perform final experiments for the chitin project.

In November 2015, she started her training in paediatrics at the department of paediatric Oncology, Haematology and Immunology at the university hospital in Duesseldorf, Germany (Prof. dr. A. Borkhardt). She will continue research on *Aspergillus*-specific T helper responses in patients during the course of stem cell transplantation funded by the 'Foundation for Investigation of Immunological and Infectious diseases' in Düsseldorf.

In 2015 Katharina married Enno Gößling.

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